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No. 1. SIMON FLEXNER, J. W. JOBLING, and MAUD L. MENTEN. Experimental studies on tumor. Plates 1–28. (Issued June 30, 1910.) Price, $1.00.

ANNOUNCEMENT

Volume VIII of *The Journal of General Physiology* will be a memorial to its founder, Dr. Jacques Loeb. This volume will contain papers by Dr. Loeb's pupils and his associates, together with a portrait and a sketch of his life. The volume will appear simultaneously with Volume IX. The publication of this volume began September 18, 1925.

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THE JOURNAL OF GENERAL PHYSIOLOGY

AVENUE A AND 66TH STREET, NEW YORK, N. Y.

STUDIES ON ENZYME ACTION.

XXXIX. Lipase Actions of Extracts of the Whole Mouse at Different Ages.

By K. GEORGE FALK and HELEN MILLER NOYES.

(*From the Harriman Research Laboratory, The Roosevelt Hospital, New York.*)

(Accepted for publication, November 17, 1926.)

INTRODUCTION.

The lipase or ester-hydrolyzing actions of extracts of the whole rat at ages from 3 days before birth until 3 years 15 days were presented

CORRECTION.

On page 263, Vol. x, No. 2, November 20, 1926, under *Curve A*, in the legend for Fig. 3, *Ordinate scale at the left* should read *Ordinate scale at the right*. Under *Curve B*, *Ordinate scale at the right* should read *Ordinate scale at the left*.

similar to several rat tumors. Similar actions were found for a number of tissues of rabbit embryos.[2] Certain mouse tumors showed actions strikingly different from those of rat tumors.[3] The question which was raised was whether mouse embryos and the youngest mice would show enzyme actions similar to mouse tumors or to rat embryos and certain rat tumors.

[1] Falk, K. G., Noyes, H. M., and Sugiura, K., *J. Gen. Physiol.*, 1925–26, viii, 75.

[2] Noyes, H. M., Falk, K. G., and Baumann, E. J., *J. Gen. Physiol.*, 1925–26, ix, 651.

[3] Sugiura, K., Noyes, H. M., and Falk, K. G., *J. Cancer Research*, 1925, ix, 129.

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INTRODUCTION.

The lipase or ester-hydrolyzing actions of extracts of the whole rat at ages from 3 days before birth until 3 years 15 days were presented in a previous communication.[1] The "pictures" of relative actions on ten esters were shown to change from a type similar to that found for the Flexner-Jobling rat carcinoma for the youngest rats to a type which may be taken to be characteristic of the adult rat and reverting to some extent for the oldest rats to the embryonic type. The absolute enzyme actions were also recorded and shown to undergo characteristic changes with increasing age of the rat.

A comparison of the ester-hydrolyzing actions of extracts of the whole rat and rat tumors with similar actions of whole mouse and mouse tumors presents a definite problem which it is the aim of this paper to answer. Rat embryos and the youngest rats showed actions similar to several rat tumors. Similar actions were found for a number of tissues of rabbit embryos.[2] Certain mouse tumors showed actions strikingly different from those of rat tumors.[3] The question which was raised was whether mouse embryos and the youngest mice would show enzyme actions similar to mouse tumors or to rat embryos and certain rat tumors.

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Experimental Methods.

Brown, and in some cases, black mice, male and female, were used. They were obtained from the stock of the Carnegie Institution of Washington, Department of Genetics, Cold Spring Harbor, Long Island, through Dr. C. B. Davenport and Miss Dorothy Newman, who made possible the satisfactory carrying out of this investigation. The mice which were used were normal as far as could be told. They were killed with ether, passed through a meat chopper twice, and water and toluene added at once. The extracts were made up with water to a volume of 25 cc. per gm. of ground material. After standing overnight, the mixtures were filtered through paper, cloudy or turbid liquids being obtained. These were brought to pH 7.0, diluted 50 per cent with water, and used for the enzyme tests. The conditions of testing the lipase actions were the same as those described previously; 15 cc. of solution, 3.4 milli-equivalents of each of the ten esters, 22 hours incubation at 37–38°, titration with 0.1 normal sodium hydroxide solution with phenolphthalein as indicator, duplicate and blank determinations, toluene present throughout. The concentrations of the mixtures tested corresponded to 26.7 mg. of original material per cc. of mixture tested.

EXPERIMENTAL RESULTS.

The experimental results will be given only for mice whose ages were definitely known. The following data include the book numbers of the experiments (for reference); the ages of the mice in days, negative values referring to number of days before birth, zero ages to the fact that the mice were killed immediately after birth; and the weights of the mice in gm., or in the experiments with very young mice where more than one was used, the average weights. The ages of those which were obtained before birth were calculated on the assumption of a gestation period of 21 days.

Book No.					5B	12B	13B	2	3	4	17
Age of mouse, *days*					−6	−6	−1	0	0	4	5
Weight " " *gm*					0.24	0.52	1.30	1.51	1.60	2.47	2.50
Book No.	6	16	9B	9A	7	36	18	8A	8B	33	19
Age of mouse, *days*	7	8	9	9	12	15	17	20	20	23	27
Weight " " *gm*	3.50	4.63	4.36	4.70	6.00	6.29	4.78	4.63	5.50	7.46	8.50
Book No.	41	22	24	27A	27B	25	28	39	40	47	48
Age of mouse, *days*	29	39	39	40	40	42	46	46	46	46	47
Weight " " *gm*	9.64	14.2	12.0	8.68	10.1	12.9	13.7	17.6	7.87	17.3	18.3
Book No.	49	23	29	50	42	30	10A	10B	51	34	35
Age of mouse, *days*	48	48	48	49	50	52	53	53	54	55	59
Weight " " *gm*	16.7	14.6	10.4	20.6	13.0	11.8	12.0	12.3	20.3	12.2	16.0
Book No.	38	32	20	1B	1A	45	26	31	44	46	11
Age of mouse, *days*	71	89	113	161	161	185	191	241	246	289	333
Weight " " *gm*	14.8	18.6	17.9	21.6	20.8	24.0	19.1	31.3	25.3	32.8	29.6
Book No.	15	43	21	37	52						
Age of mouse, *days*	437	452	466	557	626						
Weight " " *gm*	32.3	38.1	26.8	31.1	21.9						

The weights of the mice did not increase regularly with their age. Occasionally the weights differed considerably even with mice of the same age and litter. The 46 day old mouse, No. 40, was about half the weight of three other mice of the same age, due, however, to a malformation of the jaw and teeth which evidently interfered with its food intake. Autopsy showed no other abnormality.

The relative actions on the esters are shown in Fig. 1. Three ages are shown in each of the first three charts, four in each of the remaining nine. Where two or more series were studied at the same age, and the results did not differ appreciably, these are averaged and presented as one series. Where marked differences were observed for two series at the same age, both curves are given. Each curve represents a set of results at a definite age. The order of arrangement of the esters is the same as in previous papers, and, in general, a similar method of treatment of the results is employed.

A study of the curves in Fig. 1 brings out the fact that there is a difference in the "pictures" of the youngest mice in comparison with the

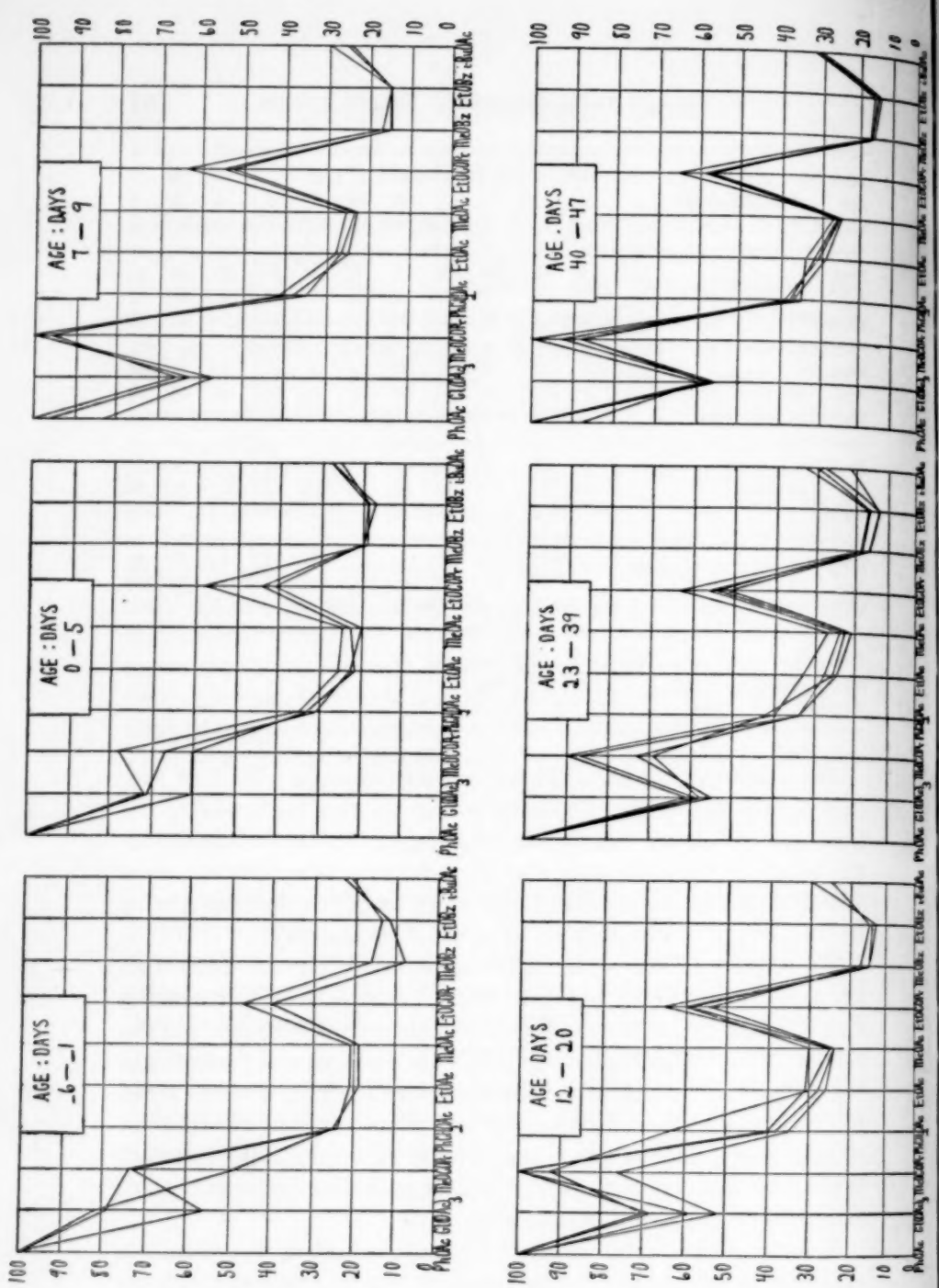

FIG. 1. Whole mice of different ages. Relative lipase actions, 45 curves, grouped in 12 plots, each curve representing the relative hydrolyzing actions of the extract of a mouse (or several mice) of definite age on the esters indicated on the abscissa axis. The ages of the mice shown in each plot are

grouped without indicating the age of the mouse for each curve. A general trend in the "pictures" of the enzyme actions with increase in age of the mouse is apparent.

oldest. The differences and changes are not as clear and definite as with the rats, but are still readily seen. The first group of curves, mouse ages 6 days before birth to 1 day before birth, approach the type found with embryo rats and a number of embryo rabbit tissues. The low benzyl acetate values, one-half to one-third those of methyl butyrate, are significant. A certain amount of irregularity is, however, apparent with these curves. In the next chart, the values of the two butyrates increase, while in the third, ages 7 to 9 days, a type which may be said to correspond to the adult mouse is apparent. This type or "picture" of the enzyme actions shows no real or constant change as the mice become older, although several changes with the oldest mice will be indicated, which, if borne out by other findings, may be rather significant.

A study of the curves shows certain irregularities in the sense that the "pictures" at times do not fall into the sequence to be expected at the indicated ages of the mice in days. There are more of such apparent exceptions than with the rats, where only isolated cases failed to conform to the general scheme. It must be remembered, however, that, in dealing with biological individuals, it is impossible to control the conditions so as to obtain chemical uniformity if such is desired, and that as with the rats the results for every mouse whose age was definitely known has been included, none being omitted because of apparent failure to conform to a preconceived scheme. As stated earlier, the mice used were not all of one strain. There is an irregularity in the sequence to which attention will be called. In the seventh chart, mouse ages 48 to 50 days, there is a real reversion toward the embryo type. The curve showing this is the average of two independent but closely agreeing series for 48 day old mice. Because of the lack of data of the longevity of the strains of mice used in these experiments, it is difficult to state with any degree of certainty whether the ages attained with the oldest mice really correspond to so called "old age".

The results answer definitely the question raised in the introduction as to whether the youngest mice correspond in type to rat embryos or to mouse carcinomas. This is brought out by the curves shown in Fig. 2, where the mouse and rat embryo "pictures" are seen to be quite similar, the rat tumor "picture" not much different, but the

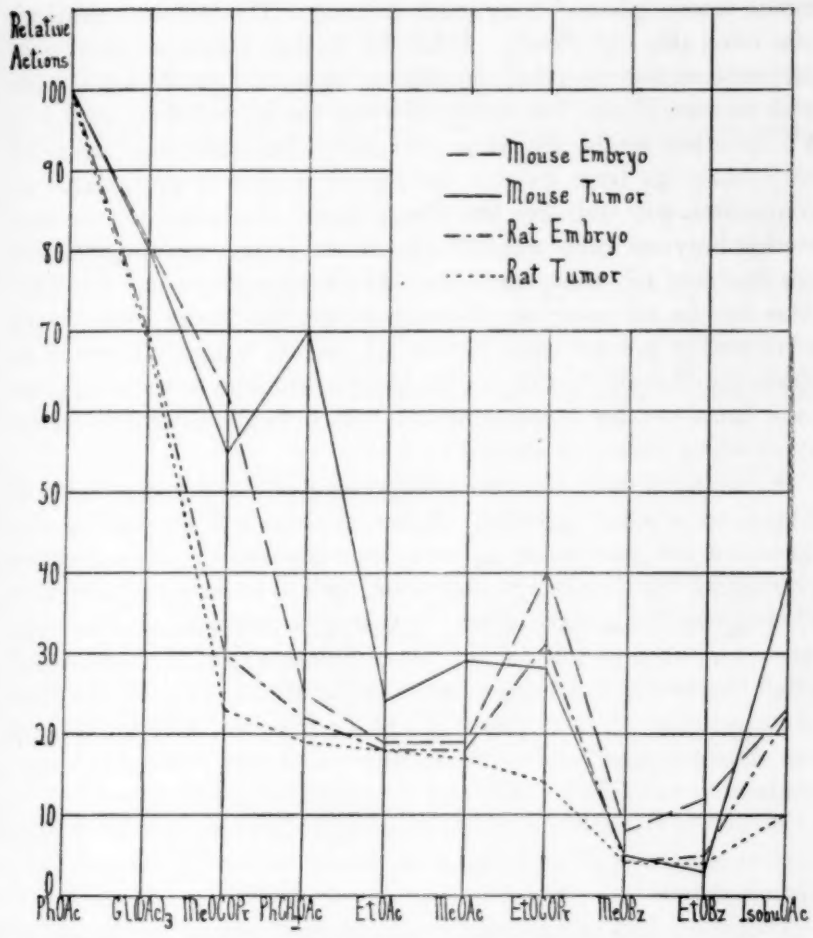

FIG. 2. The four curves of relative actions refer to the mouse embryo (6 days before birth), mouse tumor (averages of the Twort and Bashford 63 mouse carcinomas), rat embryo (averages of rats taken 3 days to 1 day before birth), and rat tumor (Flexner-Jobling rat carcinoma). The high value of benzyl acetate for the mouse tumor differentiates this curve sharply from the rest. The mouse embryo and mouse tumor have higher values for methyl butyrate than do the rat embryo and rat tumor; while the mouse and rat embryos have somewhat higher ethyl butyrate values than the mouse and rat tumors. On the whole, the curves for the mouse and rat embryos are quite similar, that for the rat tumor not greatly different, but that for the mouse tumor quite different.

mouse tumor "picture" very much different The following relations also bring this out clearly. With the methyl butyrate-benzyl acetate actions, for the mice, the ratio of these actions did not change with increase in age, but oscillated about the general mean of 2.3 to 1. In other words, the value for methyl butyrate was from 1.5 to 3 times as large as that for benzyl acetate at every age. In comparison with this, for the Twort mouse carcinoma the average methyl butyrate value was 0.93 that of the benzyl acetate, while for the Bashford 63 mouse carcinoma, the corresponding value was 0.91. Also for the rat embryos, the methyl butyrate values were always considerably greater than the benzyl acetate values. These facts prove conclusively that the mouse embryos conform to the embryonic type found in other connections and differ from the two mouse tumor types which have been studied.

A number of regularities are readily observed if the results shown in Fig. 1 are studied carefully. A few of these will be pointed out. The ethyl butyrate values are lower than the methyl butyrate values throughout the life cycle of the mouse, their average value being very close to two-thirds of the latter. This may be compared to an average value very close to 1.00 for the rats of different ages, the methyl and ethyl butyrate values being practically the same. The average ethyl acetate-methyl acetate ratio was 1.14 for mice and 1.20 for rats for the different ages, while the average ethyl benzoate-methyl benzoate ratio was 0.88 for mice and very close to 1.30 for rats.

Because of the lack of marked change of the "pictures" of the enzyme actions of mice at different ages, the results will not be discussed further in detail.

Pregnancy had no influence on the enzyme "picture" of the mother mouse. Several were tested in this way and showed the "pictures" corresponding to the age of the otherwise normal mouse. This conclusion agrees with the results obtained with pregnant rats.

The absolute values of the ester-hydrolyzing actions are shown in Table I. The results for mice of the same or nearly the same ages are averaged and presented as one series of results. The actions increased on all the esters with increase in age of the mice, beginning with comparatively small values for the embryo, reaching high values

TABLE I.

Hydrolyzing Actions in Tenths of Milli-Equivalents of Acid Produced by Extracts of Whole Mice of Different Ages on the Indicated Esters.

Age	PhOAc	Gl $(OAc)_3$	MeOCOPr	$PhCH_2OAc$	EtOAc	MeOAc	EtOCOPr	MeOBz	EtOBz	i-BuOAc
days										
−6	1.67	1.34	1.03	0.42	0.32	0.32	0.68	0.14	0.20	0.38
−1, 0, 0 (Av.)	2.69	1.77	1.85	0.77	0.59	0.56	1.15	0.49	0.46	0.69
4	3.17	1.91	1.91	1.04	0.69	0.73	1.39	0.62	0.56	0.89
5	3.25	2.33	2.53	1.15	0.83	0.80	1.90	0.61	0.62	0.83
7	4.29	2.96	5.17	1.79	1.42	1.23	2.86	0.79	0.77	1.34
8, 9, 9 (Av.)	3.70	2.42	3.69	1.50	1.00	0.94	2.25	0.61	0.55	1.07
12	4.75	2.82	4.78	1.81	1.35	1.15	2.83	0.73	0.67	
15	4.20	2.86	4.21	1.68	1.30	1.26	2.54	0.65	0.56	1.28
17	4.48	3.12	4.14	2.80	1.36	1.10	2.90	0.76		
20, 20 (Av.)	6.31	3.30	4.78	2.20	1.62	1.50	3.37	1.06	0.94	1.56
23	5.51	3.26	4.78	1.90	1.54	1.32	3.10	0.88	0.72	1.14
27	4.79	2.62	3.48	1.63	1.18	1.00	2.46	0.78	0.64	1.34
29	5.38	3.12	3.69	2.26	1.42	1.25	2.88	0.96	0.86	1.64
39, 39, 40, 40 (Av.)	5.28	3.09	4.79	2.04	1.69	1.36	3.38	0.89	0.81	1.72
42	5.69	3.78	6.45	2.24	2.21	1.75	3.79	1.10	1.00	2.09
46 (Av. of 4)	5.31	3.10	4.66	1.97	1.52	1.31	3.02	0.87	0.77	1.69
47	5.23	2.94	4.60	2.03	1.61	1.37	3.04	0.84	0.75	1.63
48 (Av. of 3)	5.21	2.85	3.49	1.95	1.19	1.08	2.46	0.76	0.64	1.39
49, 50 (Av.)	5.89	3.40	5.19	2.34	1.57	1.44	3.21	0.99	0.78	1.99
52	6.15	3.57	5.65	2.26	2.09	1.84	4.00	1.16	0.90	2.18
53 (Av. of 2)	5.75	2.85	4.91	1.89	1.43	1.09	2.94	0.92	0.78	1.45
54, 55	4.98	3.02	3.95	2.06	1.48	1.28	2.88	0.89	0.80	1.79
59	5.21	2.92	4.58	1.93	1.39	1.17	2.90	1.01	0.74	1.58
71	6.38	3.67	5.73	2.45	1.84	1.65	3.40	1.14	0.91	2.16
89	5.77	3.33	4.55	2.02	1.33	1.26	2.84	1.02	0.74	1.76
113	4.76	2.58	4.14	1.91	1.35	1.15	2.78	0.74	0.65	1.53
161 (Av. of 2)	5.91	3.26	5.93	2.59	1.78	1.48	3.44	0.87	0.85	2.02
185, 191 (Av.)	5.74	3.29	5.02	2.12	1.77	1.45	3.42	0.90	0.79	2.10
241, 246 (Av.)	5.53	3.11	5.43	2.24	1.71	1.46	3.36	0.88	0.78	2.02
289	5.57	3.51	5.84	2.29	1.88	1.67	3.48	0.92	0.87	2.09
333	6.33	2.87	4.14	2.28	1.37	1.19	2.98	0.87	0.65	1.82
437, 452, 466 (Av.)	4.70	2.78	4.56	1.82	1.42	1.25	2.83	0.85	0.71	1.56
557	5.31	2.70	3.30	1.86	1.30	1.18	2.83	0.86	0.70	1.54
626	5.86	3.08	4.14	2.05	1.42	1.23	2.64	0.86	0.66	1.74

at the age of about 7 to 20 days, and then fluctuating irregularly about these.

These results may be compared with the absolute ester-hydrolyzing actions observed with whole rats of different ages. With the rat, the actions were small for the embryo, increased rapidly with increase in age to apparent maxima, and then decreased gradually as the rats became older. The small initial values followed by increases are similar for the mouse and the rat, but the markedly decreased actions on almost all the esters, as the animals aged, which were found with the rat were not observed with the mouse. This may have been due in part, perhaps, to the fact that the ages of the oldest mice were not as great relatively as those of the rats.

SUMMARY.

The ester-hydrolyzing or lipase actions of extracts of whole mice whose ages ranged from approximately 6 days before birth to 1 year 8 months 21 days were tested on ten simple esters by the method described in previous papers. The "pictures" of the relative enzyme actions changed from a type approaching the "embryonic" as found with embryo rats and a number of tissues of rabbit embryo, to a type characteristic of the adult mouse. The mouse embryos corresponded to the rat embryos in type and differed markedly from the mouse carcinomas which have been studied. The relative and absolute enzyme actions are discussed in some detail, and the results compared with the results obtained for the life cycle of the rat.

The writers wish to thank Mr. Edmond White for assistance in carrying out the experimental work.

AMPHOTERIC BEHAVIOR OF COMPLEX SYSTEMS.

III. The Conductivity of Sulfanilic Acid-Lysin Mixtures.*

By ALLEN E. STEARN.

(From the Gates Chemical Laboratory, California Institute of Technology, Pasadena.)

(Accepted for publication, July 26, 1926.)

The hypothesis that a mixture of two ampholytes behaves, within certain limits, as an individual (1) with characteristics distinct from either component can also be semiquantitatively tested by certain conductivity experiments. These are based on the observation that the addition of such a substance as gelatin markedly decreases the observed conductivity of a phosphate solution. The magnitude of the decrease is a function of the pH and passes through a minimum at the isoelectric point of the gelatin. In other words, the specific contribution of the gelatin to the measured conductivity is negative. Consider the case of two such ampholytes, A and B. Their separate effects on the conductivities of buffer solutions will be as above, and will be a minimum at their respective isoelectric points. When mixtures of the two are observed, however, their effect is altered due to the fact that through a certain pH range, namely between their respective isoelectric points, there will also be a tendency for mutual "binding" of A and B with a resulting "release" of buffer, resulting in a measured conductivity greater than would be calculated from a knowledge of their behavior when observed separately. This difference between the observed and calculated value of the conductivity may be expected to pass through a maximum at or near what has been termed the isoelectric point of the system.

In case the substances A and B have themselves conductivities comparable with those of the buffer, there is another possibility, which is much more probable, but which cannot be certainly predicted.

* Contributions from the Gates Chemical Laboratory, California Institute of Technology, No. 117.

Their specific contributions to the conductivity, when observed separately, may be positive or negative, or may even change sign at some definite pH. One might expect, however, that, if there be any considerable mutual binding tendency between two fairly conducting substances, the measured conductivity will be less at the isoelectric point of the system than would be expected from their separate behaviors, and that this difference would be a maximum at this pH.

To test this idea the conductivities of sulfanilic acid and of lysin were measured in phosphate buffers. The buffers were in all cases 0.02 molal in phosphate. The concentration of the sulfanilic acid and of the lysin were, throughout, the same, the former being 0.020 molal and the latter about 0.023 molal. The mixtures were 0.020 molal in sulfanilic acid and 0.023 molal in lysin. The water employed in the work had a specific conductivity of about 2×10^{-6} reciprocal ohms at room temperature.

The measured conductivities at 25°C., in reciprocal ohms, are given in Table I. The pH values were measured by means of the apparatus used in the titration work described in the preceding paper (1).

From these values pH-conductivity curves were plotted and the conductivities at comparable pH values were read off. These latter values are given in Table II. Obviously the same buffer mixture will not bring sulfanilic acid to the same pH that it will lysin when the buffer concentration is at all comparable to that of the sulfanilic acid or the lysin. It will be seen, however, that for any pH, with constant total phosphate concentration, the concentrations, and thus the conductivities, of the various anion species of the buffer will be the same in all cases unless certain of them tend to be bound by the sulfanilic acid or the lysin. There will, however, be a difference in the sodium ion concentration, and some correction must be made for this. In Table II there is therefore included the total sodium concentration. The correction is made by referring to the concentration in the pure buffer at the same pH. For example, at a pH of 4 there was a concentration of 0.0197 in the buffer but of 0.0464 in the sulfanilic acid. To get the contribution of the sulfanilic acid itself, the measured conductivity of the buffer is subtracted from that of the sulfanilic acid and buffer at the same pH. This resulting conductivity is partly due to the sulfanilic acid, and partly due to the excess of sodium ion.

TABLE I.

Buffer		Sulfanilic acid plus buffer		Lysin plus buffer		Mixture plus buffer	
pH	*k*	*pH*	*k*	*pH*	*k*	*pH*	*k*
3.77	590	3.75	1105	3.75	543	3.90	1078
4.04	591	4.50	1134	4.24	564	4.34	1104
4.37	590	5.72	1200	4.87	592	4.74	1130
6.28	718	5.85	1208	5.26	630	5.30	1165
6.78	833	6.33	1251	5.65	709	5.75	1221
7.27	936	6.63	1306	5.88	762	5.91	1220
7.77	995	6.88	1355	6.36	831	6.00	1214
8.70	1010	7.12	1400	6.83	932	6.21	1230
9.75	1026	7.57	1457	7.40	1002	6.41	1287
10.27	1049	7.93	1487	7.95	1044	6.61	1345
10.70	1090	8.68	1510	8.43	1065	6.83	1383
11.07	1175	10.20	1550	8.83	1080	7.03	1405
		10.40	1565	9.23	1110	7.27	1434
		11.07	1677	9.72	1162	7.83	1496
				10.42	1270	8.36	1539
				11.00	1425	8.62	1555
						9.42	1622
						10.10	1717
						11.12	1965

TABLE II.

pH	Buffer		Sulfanilic acid			Lysin			Mixture		
	Na	*k*	*Na*	*k*	*k'*	*Na*	*k*	*k'*	*Na*	*k*	*k'*
4.0	.0197	590	.0464	1117	527	.0137	554	−36	.0403	1085	495
5.0	.0207	613	.0484	1166	553	.0153	601	−12	.0428	1147	534
5.2	.0209	623	.0485	1175	552	.0158	624	1	.0431	1164	541
5.4	.0215	635	.0485	1180	545	.0168	645	10	.0438	1170	535
5.6	.0220	649	.0486	1190	541	.0176	694	45	.04415	1196	547
5.8	.0231	665	.0496	1205	540	.0192	756	91	.0452	1226	561
6.0	.0243	684	.0506	1224	540	.02115	771	87	.0465	1214	530
6.2	.0258	706	.05144	1236	530	.0232	800	94	.0479	1228	522
6.4	.0276	739	.0525	1262	523	.0259	838	99	.0498	1286	547
6.6	.0297	785	.0537	1300	515	.0290	890	105	.0518	1344	559
6.8	.0320	835	.05525	1338	503	.0312	929	94	.0537	1382	547
7.0	.0343	880	.0570	1375	495	.0336	955	75	.0557	1402	522
8.0	.0393	1002	.0612	1489	487	.0389	1046	44	.0605	1511	509
9.0	.040	1013	.0625	1514	501	.0429	1090	77	.0645	1578	565
10.0	.0405	1035	.0632	1538	503	.0472	1199	164	.0695	1701	666
11.0	.0436	1165	.0660	1666	501	.0556	1425	260	.078	1933	768

The values of the conductivities must be multiplied by 10^{-6} to give reciprocal ohms.

The difference in total sodium concentration can be at once obtained, but some assumption must be made as to the relation of the total sodium concentration to that of the sodium ion. Due to the necessity of this correction, the values of these conductivities are not presented as significant data in themselves, in fact their precise magnitude as well as their real significance is a question. However, the general shape of the curve obtained by plotting them against the pH is considered significant, and slight errors in the sodium ion correction will alter neither the general shape of the curve nor the position of the

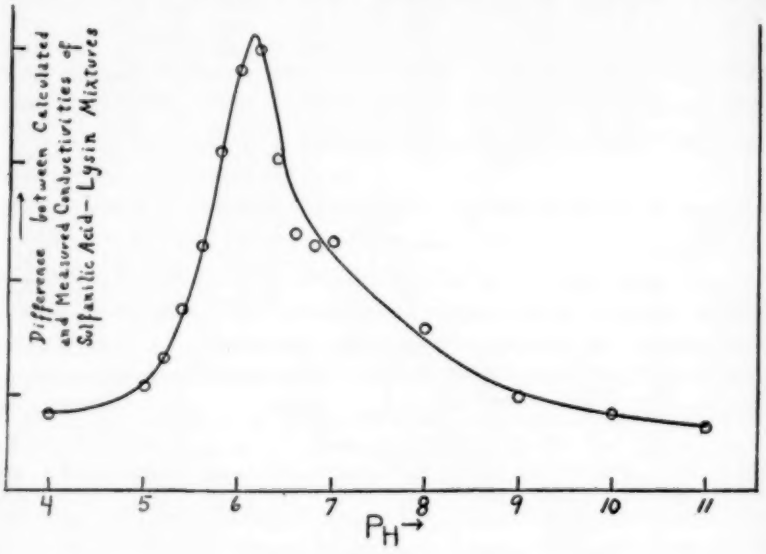

FIG. 1.

maximum in Fig. 1. Assuming, then, the isohydric principle, and the value 0.92 for the degree of ionization of the sodium salts, the conductivity correction for the sodium ion can be calculated from its ion conductance at 25°C., 50.7, and the cell constant. Table II, then, gives the total sodium concentration, the measured total conductivity in the column headed k, and the apparent conductivity of the substances studied, uncorrected for sodium ion concentration difference, in the column headed k'. The latter value is merely the difference between the conductivity of the solution and that of pure buffer at the same pH.

Table III gives the final corrected values for the contributions of sulfanilic acid, lysin, and their mixture to the total conductivity. The last column contains the differences obtained by subtracting the measured contribution of the mixture from the sum of the contributions of the two components when studied separately. These differences are plotted against pH in Fig. 1.

This curve, representing the pH function of the decrease in conductivity from what might be expected, due presumably to mutual binding of sulfanilic acid and lysin, passes through a fairly well defined

TABLE III.

pH	$k \times 10^6$				
	Sulfanilic acid	Lysin	Mixture (observed)	Mixture (calculated)	Difference
4.0	157	48	209	205	−4
5.0	167	63	227	230	3
5.2	169	72	231	241	10
5.4	170	75	225	245	20
5.6	172	106	239	278	39
5.8	172	145	254	317	63
6.0	175	131	222	306	84
6.2	174	130	215	304	89
6.4	177	123	239	300	61
6.6	179	115	252	294	42
6.8	180	105	246	285	39
7.0	180	85	225	265	40
8.0	183	50	215	233	18
9.0	188	37	225	225	0
10.0	188	71	263	259	−4
11.0	190	93	290	283	−7

maximum somewhere between the pH values 6.1 and 6.2. Calculation, by the method described in the preceding paper (1), of the value of the isoelectric point of this system, gives, using 7×10^{-4} for the acid ionization constant of sulfanic acid and 7×10^{-8} for the basic ionization constant of lysin, a pH of 6.03. The agreement seems quite satisfactory, considering the method of obtaining the experimental data and the problematical value of the basic ionization constant of lysin.

DISCUSSION.

While the above experiments were made on a comparatively simple system in order that a somewhat more definite interpretation might be possible, the more interesting and perhaps more obvious applications of the results are in connection with the much more complicated, though in many respects similar, systems which go to make up biological tissues.

One of the striking apparent anomalies which the point of view developed in this series of papers tends to straighten out is brought out in Fig. 2. From water absorption and behavior toward dyes Robbins (2) has found for the complex system potato tuber an isoelectric point at a pH of about 6, depending somewhat on the buffer used for adjusting the pH (Curve *C*). That this value is not even approximately characteristic of the protein most commonly associated with potato, namely tuberin, is apparent from the work of Cohn, Gross, and Johnson (3), who found for this protein an isoelectric point at a pH of about 4. Their tuberin was obtained from acid precipitation of potato juice. It is significant to note that they describe the precipitation of protein from potato juice by alkali as well as by acid. The latter precipitation reached a maximum at a pH of about 8, but was not otherwise studied. The solubility curve for the protein material in potato juice as a function of pH is given by Curve *A* of Fig. 2. (Curve *B* gives the same for carrot juice indicating similar behavior.) Both are taken from the work of Cohn, Gross, and Johnson. The point of maximum solubility between the two minima corresponds roughly with Robbins' isoelectric point of the system potato tuber, *i.e.* with the point of minimum water imbibition (Curve *C*). The two points are not exactly the same, but Robbins was working with whole tissue, while Cohn, Gross, and Johnson were working with the extracted juice.

The comparatively large specific effect of the particular buffering material employed on the isoelectric point of a complex system may also be expected, due to selective "binding tendencies" between the specific buffer ions and one or another of the components of the original system. Thus Robbins (2) finds a difference of nearly half a pH unit between the isoelectric points of potato tuber tissue as determined

by using citrate or phosphate buffer and as determined by phthalate buffer. In Fig. 2, Curve *C*, Curve I was obtained using phosphate adjustments and Curve II using phthalate adjustments.

The concept of such a mixed system offers also a possible chemical mechanism for the taking on of foods of both a basic and an acidic

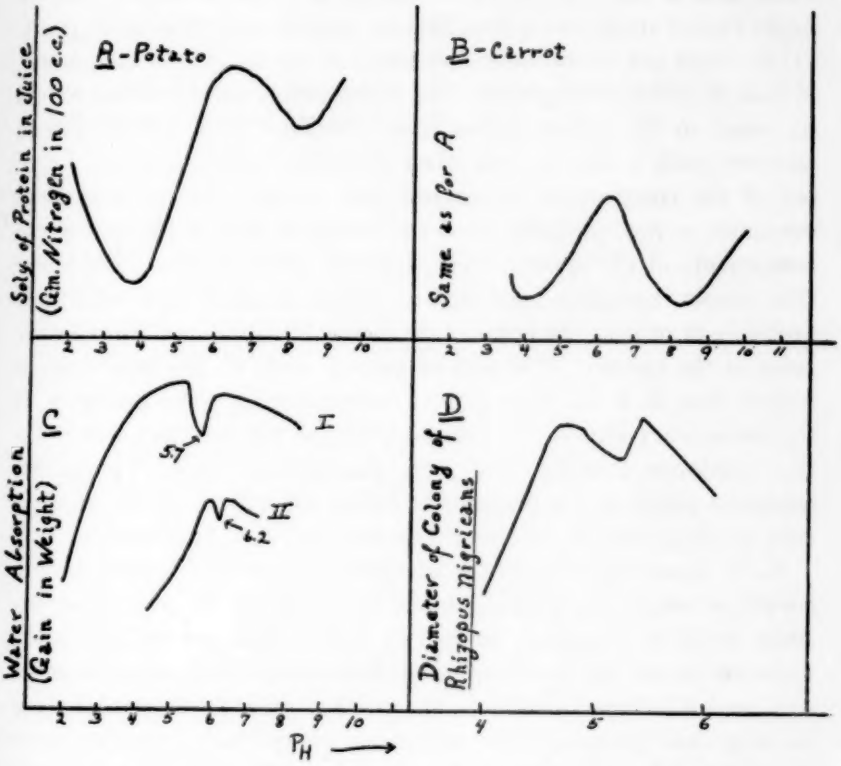

FIG. 2. Curve *A*, solubility of protein material in potato juice; Curve *B*, solubility of protein material in carrot juice (3); Curve *C*, water absorption by potato tuber tissue (2); Curve *D*, growth curve of *Rhizopus nigricans* on potato dextrose agar (4).

nature. Thus, in the case of a complex system, there will be a fair pH range through which one or the other of the components will exhibit a tendency to bind foods either of a carbohydrate or of a peptone nature. Growth curves over a fair pH range are suggestive on this point

(Fig. 2, Curve *D*) (4). At quite low or quite high pH values, nutrition, according to these curves, is very inefficient. Starting in acid solution, as the pH increases the rate of growth at first steadily increases. If the taking on of foods is primarily influenced by the ionic condition of the organism rather than by the ionic condition of the foods, and if the organism were acting as a simple ampholyte, we might expect an optimum condition for growth at its isoelectric point. (This would not be analogous to water imbibition.) At such a pH, in case of a simple ampholyte, the active anion concentration would be equal to the active cation concentration. In a mixed system, however, such is not the case when the lower isoelectric point, *i.e.* of one of the components, is reached, and actually the growth curve continues to rise, probably until the extent of mutual binding of the components of the system itself begins to affect results. The curve thus passes through a maximum and then descends to a minimum, probably at or near the point of maximum binding, *i.e.* the isoelectric point of the system. The rate of growth, even at this minimum, is higher than it is at those points corresponding more probably to the isoelectric points of the components, and the fact that it is a distinct minimum does not at all mean that growth is poor. From this minimum point, as one proceeds to higher pH values, the curve again rises, passing through another maximum, and then rapidly falls.

Work is now in progress to determine, if possible, the pH growth curves of organisms utilizing foods which might be considered entirely acidic in character, as well as foods which are entirely basic. Experiments on the specific effect of individual buffers are also under way, and it is hoped that soon the point of view developed here can be somewhat quantitatively tested out on systems somewhat more complicated than those herein described, but which are still sufficiently definitely known to permit of quantitative study and interpretation.

SUMMARY.

Conductivities of sulfanilic acid, lysin, and mixtures of the two were made over a wide pH range, the pH being adjusted by means of phosphate buffers. The actual conductivities of the sulfanilic acid, the lysin, and the mixture were calculated. The difference between the conductivity of the mixture and the sum of the conductivities of

the components alone passes through a maximum at a pH theoretically calculable as the isoelectric point of the system.

Certain applications of the results are made to the explanation of the behavior of living tissues.

BIBLIOGRAPHY.

1. Stearn, A. E., *J. Gen. Physiol.*, 1926–27, x, 313.
2. Robbins, W. J., *Am. J. Bot.*, 1923, x, 412.
3. Cohn, E. T., Gross, J., and Johnson, O. C., *J. Gen. Physiol.*, 1919–20, ii, 145.
4. Robbins, W. J., *J. Gen. Physiol.*, 1923–24, vi, 259.

AMPHOTERIC BEHAVIOR OF COMPLEX SYSTEMS.

IV. Note on the Isoelectric Point and Ionization Constants of Sulfanilic Acid.*

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(Accepted for publication, July 26, 1926.)

In the experiments described in the preceding papers the assumption was made that sulfanilic acid was an ampholyte. While its formula would tend to justify such an assumption, there seems to be no record of any determinations of its basic ionization constant or of its isoelectric point. It is to be expected that the magnitude of the former is very small and that the isoelectric point will be at a very low pH.

Titration methods do not offer a satisfactory method of attack since the pH at which basic neutralization commences is so low that a small change in E.M.F. corresponds to a relatively large difference in hydrogen ion concentration, and moreover the hydrogen ion concentration change is not a measure of salt formation in such a region of pH. Also the large acid ionization constant of the sulfanilic acid tends to "cover" or mask any small basic neutralization.

Solubilities were therefore determined in water and in various concentrations of hydrochloric acid. Table I gives results at 24°C., which are typical. The first eight columns give the molalities of the substances named. The basic ion from the sulfanilic acid is called S^+, and the acid ion S^-. K, in Column 9, is the function $\frac{(H^+)\left(\begin{matrix}\text{unionized}\\ \text{sulfanilic}\end{matrix}\right)}{(\text{salt})}$. The degree of hydrolysis, h, is calculated for a salt concentration equal to the concentration of HCl corresponding to its place in the table.

* Contributions from the Gates Chemical Laboratory, California Institute of Technology, No. 118.

Values of h are calculated by taking a total salt concentration equal to the corresponding hydrochloric acid concentration and, using the value of K in Column 9, solving for the concentration of unhydrolyzed

TABLE I.

1 HCl	2 $H^+ \times 10^3$	3 $OH^- \times 10^{-3}$	4 Solubility of sulfanilic acid	5* $S^- \times 10^4$	6† $S^+ \times 10^4$	7 Unionized sulfanilic acid	8‡ Salt $\times 10^4$	9§ K	10‖ h	11‖ $K_h \times 10^{11}$
.00	.67	15.	.0715	56.	.93	.0648	—	—	—	—
.05	4.7	2.1	.0664	9.8	6.3	.0648	—	—	—	—
.07	6.5	1.54	.0661	7.0	8.5	.0646	—	—	—	—
.08	7.4	1.35	.0663	6.2	9.6	.0647	—	—	—	—
.087	8.0	1.25	.0665	5.8	10.5	.0649	—	—	—	—
.10	9.2	1.08	.0675	5.1	—	—	1.72	3.46	.97	2.6
.25	22.5	.44	.0689	2.1	—	—	3.77	3.92	.948	2.3
.50	42.	.24	.0709	1.2	—	—	5.9	4.6	.923 (.914)	1.8 (2.1)
.85	68.	.147	.0714	.73	—	—	6.5	6.75	.917 (.874)	1.2 (1.95)
Mean						.06476				
1.			.0713							
1.3			.0705							
1.8			.0668							
2.			.0659							
2.5			.0645							
4.			.058							
6.			.051							

* Using the value 7×10^{-4} for the acid ionization constant.

† Using the value 2×10^{-15} for the basic ionization constant.

‡ Obtained by subtracting the mean of the values in Column 7 plus twice the respective values in Column 5 from the values in Column 4.

§ The indicated function of the values in Columns 2 and 8 with the mean of those in Column 7.

‖ The values in parentheses are obtained by putting K (Column 9) equal to 4.0. See text.

chloride. We are in such a pH region, however, that though we can neglect the acid ionization of sulfanilic acid, we cannot neglect the ionization of the HCl formed by hydrolysis; so that, since the hydrolysis is in all cases large, the measured value of the hydrogen ion con-

centration for the corresponding molality of HCl is substituted in the formula rather than the total concentration of the HCl formed by hydrolysis.

It will be noted that the values of K in Column 9 regularly increase. This is due to the fact that at higher concentrations of HCl the actual solubility of the sulfanilic acid decreases rapidly as the HCl concentration increases, as shown in Table I. At these higher HCl concentrations we are in such a pH region that the thermodynamic environment of the solvent changes rapidly with comparatively small changes in the ratio of the hydrogen ion concentration. For this reason it is thought that a value of about 4.0 for K in Column 9 is probably not far from correct, and the corresponding values of h and K_b are accordingly given in parentheses.

An approximate check on the magnitude of K_b can be obtained by noting the fact that there is a point of minimum solubility which comes apparently between cH values 0.047 and 0.065. Using the well known Michaelis relation, and the value 7×10^{-4} for K_a, K_b is found to lie between 1.7 and 3.2×10^{-15}.

Other Constants Used in the Series.

From the titration curves in Paper II of this series the pH at which any fraction of glycine or sulfanilic acid is neutralized may be read. This permits a calculation of their ionization constants, values of which are given in Table II.

These values are in good agreement with those given by Scudder (1) for glycine. The curve for sulfanilic acid did not permit as satisfactory reading, and since the literature gives values which seem to vary somewhat, the following determination of the acid ionization constant of sulfanilic acid was made. The method was to take conductivity ratios at different known concentrations. Since conductivities are additive, their ratios will be a measure of the ratios of the sulfonate ion concentrations at respective total acid concentrations. Thus if X be the concentration of sulfanilic ion (and also of hydrogen ion) at total concentration C, and X' be their concentrations at total concentration C', then $X' = rX$, where r is the ratio of the conductivities at concentrations C and C'.

TABLE II.

Ion/molecule ratio	Glycine				Sulfanilic acid	
	$cOH \times 10^{12}$	$K_b \times 10^{12}$	$cH \times 10^{10}$	$K_a \times 10^{10}$	$cH \times 10^{4}$	$K_a \times 10^{4}$
1 : 3	6.45	2.15	6.6	2.2	—	—
1 : 2	4.26	2.13	4.46	2.23	14.1	7.05
1 : 1	2.24	2.24	2.24	2.24	7.24	7.24
2 : 1	1.1	2.2	1.15	2.3	—	—
3 : 1	.76	2.28	.79	2.37	—	—
Mean.....		2.2		2.27		7.15

TABLE III.

Bottle	Total concentration sulfanilic acid	Resistance	Conductivity $\times 10^{6}$
1	.05	1736	576.0
2	.01	4175	239.5
3	.005	6234	160.4
4	.0025	9513	105.1
5	.001	17391	57.5

Combination	Ratio	$X \times 10^{3}$	$K_a \times 10^{4}$
1–2	2.405	2.321 sulfanilic ion concentration Bottle 2	7.02
1–3	3.591	1.5565 " " " " 3	7.04
1–4	5.4805	1.021 " " " " 4	7.05
1–5	10.02	.5576 " " " " 5	7.03
2–3	1.493	1.5565 " " " " 3	7.04
2–4	2.279	1.0235 " " " " 4	7.10
2–5	4.165	.5573 " " " " 5	7.02
3–4	1.526	1.024 " " " " 4	7.11
3–5	2.790	.5606 " " " " 5	7.16
4–5	1.828	.5561 " " " " 5	6.97
Mean.....			7.05

The value 7×10^{-4} has been used throughout.

One can thus obtain the two equations;

$$\frac{X^2}{C - X} = K, \quad \text{and} \quad \frac{(rX)^2}{C' - rX} = K$$

which can be solved simultaneously for X and K.

Table III gives the values obtained. The water used had a specific conductivity of 3.5×10^{-6} reciprocal ohms, and the temperature was 21.00°C. ± 0.02°, since it was at that temperature that the titration work had been largely carried out.

SUMMARY.

From the solubility minimum the value of the basic ionization constant of sulfanilic acid is shown to lie probably between the values 1.7×10^{-15} and 3.2×10^{-15}. From solubility measurements the value of this same constant is shown to lie probably between 2.0 and 2.2×10^{-15}, and the isoelectric point of sulfanilic acid is thus at a cH of 0.056 or a pH of 1.25. From conductivity ratios the acid ionization constant of sulfanilic acid is shown to be 7.05×10^{-4} at room temperature (21°C.).

Calculations are made, from data published in preceding papers, of the ionization constants of glycine, K_a being 2.3×10^{-10}, and K_b being 2.2×10^{-12}.

BIBLIOGRAPHY.

1. Scudder, H., Electrical conductivity and ionization constants of organic compounds, New York, D. Van Nostrand Co., 1914.

THE OXIDATION-REDUCTION POTENTIAL OF THE LUCIFERIN-OXYLUCIFERIN SYSTEM.

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(Accepted for publication, October 27, 1926.)

Oxygen is necessary for the luminescence of the ostracod crustacean, *Cypridina*, and most luminous animals. A substance, luciferin, is oxidized to oxyluciferin in the presence of a catalyst, luciferase. The oxyluciferin can be reduced to luciferin again, so that one is justified in speaking of a luciferin $\rightleftharpoons$ oxyluciferin system. When the words luciferin, oxyluciferin, and luciferase are used in this paper, I refer only to the crude impure solutions from *Cypridina hilgendorfii*. For the preparation and isolation of these substances the reader is referred to my book (1920) or earlier papers (1919, *b*).

We should like to know more about the nature of the oxidation of luciferin. First, it must be understood that the word oxidation is used for a number of quite different processes. We may recognize at least two distinct types of *reversible* oxidations.

(1). The oxidation of hemoglobin to oxyhemoglobin, in which the oxygen is actually bound, but very loosely. This well known change depends on the concentration or pressure of the oxygen and is more properly called an oxygenation than an oxidation. Such oxygenations do not possess an oxidation-reduction potential. Luciferin is not to be compared with hemoglobin because removal of the oxygen from an oxyluciferin solution will not bring about the formation of luciferin, as it will the formation of hemoglobin from oxyhemoglobin.

(2). The oxidation of hemoglobin to methemoglobin, in which no oxygen is actually bound in the methemoglobin molecule although oxygen may be used up in its formation. However, the reaction may go on in complete absence of oxygen, by oxidation with $K_3Fe(CN)_6$. Conant (1923–25) and his coworkers have thrown new light on this system, which indicates that it has a definite oxidation-reduction po-

tential. If hemoglobin in acid solution is represented by $H_3Hb^{\equiv}$, then oxyhemoglobin is $H_3HbO_2^{\equiv}$ and methemoglobin is $H_2Hb^{=}$. A change in valence is the essential difference in the hemoglobin-methemoglobin transformation.

Other oxidations in which the essential change is one of valence necessitate also the removal of hydrogen, such as the oxidation of a leuco dye to the dye itself. Oxygen plays the rôle of hydrogen acceptor, forming water. Thus, methylene white (leucomethylene blue), MH_2, and safranin white (leucosafranin) oxidize to methylene blue, M, or safranin spontaneously in the air by loss of hydrogen;— $MH_2 + O = M + H_2O$. As Clark and his coworkers (1923–26) have shown, these reactions have a definite oxidation-reduction potential also.

I have previously pointed out (1918–19, *a*) that the oxidation of luciferin bears some resemblance to the oxidation of a leuco dye and presumably has an oxidation-reduction potential. The equation might be represented thus:

$$LH_2 \text{ (luciferin)} \rightleftharpoons L \text{ (oxyluciferin)} + H_2$$
$$H_2 + O = H_2O.$$

For a more complete discussion of oxidation-reduction potential the reader is referred to the papers of Clark and collaborators (1923–26), Conant (1926), and Conant and Cutter (1924). Suffice it to say here that oxidizing and reducing agents can be arranged in a series of varying strength, with strongest reducing agents at one end and strongest oxidizing agents at the other. The strength is conveniently measured by the potential which the oxidizing or reducing agent gives in contact with some noble indifferent metal, like gold or platinum. Reproducible potentials are observed only if the reaction (reductant $\rightleftharpoons$ oxidant) is reversible, and the exact value varies with the H ion concentration and the ratio of oxidized to reduced substance. The symbol, E_o', is used for the potential of such an oxidizing agent as methylene blue at a definite pH and with chemically equivalent amounts ([red.]/[oxid.] = 1) of methylene blue (oxidant) and methylene white (reductant) in solution. The accompanying table gives the E_o' values for a number of oxidizing or reducing agents at a pH = 7.7, a favorable value for luminescence of luciferin.

The H electrode represents the reducing power of hydrogen gas under one atmosphere pressure in presence of a catalyst like finely divided platinum. Many reducing agents like sulfides and chromous chloride stand above the H electrode and hydrosulfites and titanous salts are near it. The oxygen electrode represents the oxidizing power of oxygen under conditions similar to those of the H electrode. Many oxidizing agents like permanganates and dichromates come below the oxygen electrode.

TABLE I.

E'_o Value at pH = 7.7.

H_2 electrode	−.46
$Na_2S_2O_4$ (Na hydrosulfite or hyposulfite)	−.32 (approximately)
Safranin	−.30 "
Anthraquinone β Na sulfonate	−.26
Anthraquinone 2-6-di Na sulfonate	−.22
Indigo monosulfonate	−.20
" disulfonate (indigo carmine)	−.15
" trisulfonate	−.115
" tetrasulfonate	−.075
Methylene blue	−.01
1-4-napthoquinone	+.03
Lauth's violet	+.04
Methemoglobin (anaerobic)	+.08 (approximately)
1-2-napthoquinone	+.13 "
2-6-dichlorindophenol	+.20
Quinhydrone (equimolecular hydrochinone and quinone)	+.24
K ferricyanide	+.43
O_2 electrode	+.76

Where does the luciferin-oxyluciferin system stand in this scale? One may gain some idea of the position of the luminescent system in the potential scale by finding what substances, whose potentials are known, will oxidize luciferin or reduce oxyluciferin in absence of oxygen. We know that oxygen and platinum will oxidize luciferin and that hydrogen and platinum will reduce oxyluciferin (Harvey, 1923), so that the position of luciferin-oxyluciferin must lie between the oxygen and hydrogen electrodes. Some years ago (January, 1922) I attempted to oxidize luciferin *with light production in absence of oxygen* by cystine, methylene blue, quinone, potassium ferricyanide,

and other substances, but always with negative results, as far as the appearance of luminescence is concerned.

Recently I have confirmed these results. I have also carried out a more systematic study to limit the potential of the luciferin-oxyluciferin system. It appears that luciferin may be readily oxidized in absence of oxygen *although no luminescence occurs*. It is all important to distinguish between the oxidation of luciferin with luminescence and without luminescence. Oxidation without luminescence may occur in absence of gaseous oxygen or in absence of luciferase (with gaseous oxygen)[1] but oxidation with luminescence never occurs without oxygen and without luciferase. I shall return to this point later.

Rapid oxidation of luciferin without luminescence can be demonstrated very clearly and simply by adding to a luminescent mixture of luciferin and luciferase in phosphate buffer (pH = 7.7), weak $K_3Fe(CN)_6$ solution drop by drop. On sufficient addition of $K_3Fe(CN)_6$ the luminescence will disappear *without any previous increase in brightness*. All the luciferin has been oxidized by the ferricyanide. On now adding weak sodium hydrosulfite ($Na_2S_2O_4$) solution to the mixture and then shaking with air, the luminescence will return. $Na_2S_2O_4$ has reduced the oxyluciferin formed by the ferricyanide, and with luciferase and oxygen present light appears. The oxidation and reduction can be brought about many times by successive additions of ferricyanide and hydrosulfite, so there can be no possibility that these reagents injure the luciferase.

It is obvious that one might titrate luciferin quantitatively with ferricyanide, using luminescence with luciferase as the end-point indicator, but some experiments along these lines show that there is no sharp end-point and lead me to believe that little significance is to be attached to such figures until luciferin can be obtained in the

[1] The autoxidation of luciferin by oxygen in absence of luciferase might be compared with the autoxidation of benzaldehyde by oxygen. It is interesting to note in this connection that small amounts of hydrochinone or diphenylamine, which so markedly prevent (negative catalysis) the oxidation of benzaldehyde, have practically no inhibiting effect on the oxidation of luciferin, as judged by the luminescence. Quinone in the hydrochinone accelerates the spontaneous oxidation of luciferin. KCN, which inhibits respiratory oxidations, does not affect the luminescence of luciferin.

pure state. There are probably other easily oxidizable substances in my solutions besides luciferin.

The experiment with ferricyanide and hydrosulfite shows at once that the potential of luciferin-oxyluciferin lies between these two substances. Since the oxidized form of any substance lower in the series of Table I will oxidize the reduced form of anything above, while the reduced form of anything above will reduce the oxidized form of anything below, we have only to test oxidation of luciferin and reduction of oxyluciferin by appropriate agents in the table.

There is only one point to guard against. If two substances lie close together in potential, the reaction between them will not be complete, for the potentials given are for equimolecular parts of oxidant and reductant, [red.]/[oxid.] = 1. For a ratio of [red.]/[oxid.] = 1/99 the potential would be shifted 50 millivolts toward the positive side, while for a ratio of [red.]/[oxid.] = 99, the potential would be shifted 50 millivolts toward the negative side. There is thus a certain band, about .1 volt wide, where oxidation and reduction will not be complete. By selecting substances in Table I fairly well separated from each other, we can limit the potential of luciferin. I have taken quinhydrone, 2-6-dichlorindophenol, 1-4-napthoquinone, methylene blue, indigo tetrasulfonate, indigo disulfonate, anthraquinone β Na sulfonate, anthraquinone 2-6-di Na sulfonate, and safranin and carried out the experiments in the following manner.[2]

Two test-tubes, *A* and *B*, are connected as shown in Fig. 1 by glass tubes through rubber stoppers. The tube, *C*, has an enlargement at *D* holding a small perforated platinum disk above which asbestos may be packed to serve as a filter. In testing for reduction of oxyluciferin, oxyluciferin + luciferase in M/10 phosphate (pH = 7.7) buffer is placed in *B* and the reducing substance, say dilute safranin in M/10 phosphate (pH = 7.7) buffer + some platinized asbestos in *A*. A stream of pure hydrogen freed of oxygen by passage over red hot platinized asbestos in a quartz tube, is then passed through the tubes, *A* and *B*, for ½ hour or more. The safranin is reduced to colorless safranin white by the hydrogen-platinum and all oxygen driven

[2] I am deeply indebted to Dr. Keith Cannan for samples of quinhydrone, dichlorindophenol, and indigo tetrasulfonate and to Dr. J. B. Conant for samples of the napthoquinones and the anthraquinone sulfonates.

from the system. Then Tube *A* is inverted and its contents filter into *B*, the platinized asbestos being kept back by the asbestos filter. This is important since oxyluciferin is reduced by hydrogen-platinum.

After mixing, oxidation of safranin will be indicated by reddening of the safranin white in the hydrogen atmosphere and reduction of oxyluciferin will be indicated by luminescence when air is admitted to the tube. There is undoubtedly some reduction of oxyluciferin by reduced safranin (which becomes red in the hydrogen atmosphere after mixing) and the anthraquinones, since luminescence is obtained when air is admitted to the mixture in *B*. A control tube of oxyluciferin and luciferase, but without addition of safranin white, or reduced anthraquinones in a hydrogen atmosphere gives no luminescence when

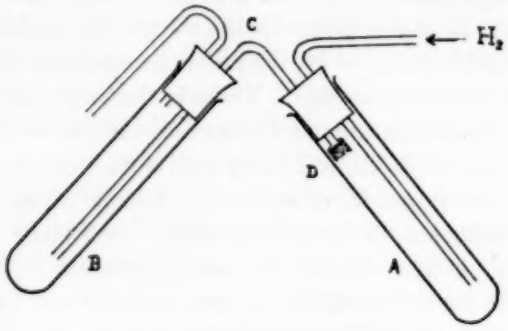

FIG. 1.

air is admitted. Of the remaining dyes, indigo disulfonate, tetrasulfonate, methylene blue, 1-4-napthoquinone, and dichlorindophenol bring about practically no reduction of oxyluciferin. This is the case when the solutions have been in contact for 5 minutes before admitting air. If allowed to stand 90 minutes before testing, reduced indigo tetrasulfonate may reduce slightly, as judged by a very faint luminescence. However, the reduced yellowish indigo tetrasulfonate does not become appreciably colored (as would occur if it was oxidized by oxyluciferin) even after 90 minutes contact, so that the slight reduction of oxyluciferin can be detected only because a very small amount of luciferin gives a sufficiently bright luminescence to be detected by the eye. In other words the luminescent test is an extremely delicate

one. I am somewhat in doubt as to the meaning of very faint luminescences since a few bacteria may have brought about the slight reduction on standing 90 minutes. Relying on experiments which show undoubted reduction, I am inclined to place the luciferin-oxyluciferin system in the region between anthraquinone 2-6-di Na sulfonate ($-.22$) and indigo disulfonate ($-.15$).

Attacking the problem from the other side, that of oxidation, we place in Tube *B* luciferin in phosphate buffer (pH = 7.7) and in Tube *A* oxidized dye in phosphate buffer (pH = 7.7). Tube *C* need not be a filter in this case. After a stream of pure hydrogen has been passed for $\frac{1}{2}$ to 1 hour, *A* is mixed with *B*. If oxidation of luciferin has occurred, the dye should become colorless and the luciferin give no more light on mixing with luciferase in air. The latter change is the best test since other reducing substances than luciferin may be present in crude luciferin solution. Passing up the list of substances in Table I from the oxidized end, there is no doubt that quinhydrone will oxidize luciferin in absence of oxygen but dichlorindophenol has practically no action and 1-4-napthoquinone or methylene blue none. Judging from these experiments on oxidation of luciferin, the potential of the luciferin-oxyluciferin system would be somewhere between dichlorindophenol ($E_o' = + .2$) and quinhydrone ($E_o' = + .24$).

The attempt to approach the potential from the oxidation and reduction side gives rather divergent values. There is thus no very sharp potential that can be assigned to luciferin. By this method of "bracketing" we find the limits for a pH = 7.7 somewhere between $-.22$ and $+ .24$ volt. Systems between these values neither reduce oxyluciferin nor oxidize luciferin. Perhaps that is due to poising[3] action in a mixture of oxidizable and protein substances, for it must not be forgotten that luciferin solutions contain many other substances from the luminous animal besides luciferin.

If impurities are not obscuring the result, the luciferin-oxyluciferin system behaves like some of those described by Conant, which are irreversible. I say irreversible, although it is quite certain that the stronger reducing agents which lie near the hydrogen electrode will reduce readily. These include hydrosulfites, sulfides, chromium chloride ($CrCl_2$), titanous chloride ($TiCl_3$), and hydrogen formed at

[3] Similar to buffer action in acid-alkaline solutions (see Clark, 1923–26).

cathodes or from metals (aluminum amalgam and magnesium in ammonium salts are especially good methods) or in contact with Pt or Pd. At the same time it is just as certain that quinone, K ferricyanide, Br water, and strong oxidizing agents will oxidize luciferin. We are not dealing with such irreversible oxidations, as in the case of aliphatic aldehydes to acids, where the acid cannot be reduced to aldehyde no matter how strong the reducing agent; or such irreversible reductions, as in the case of dibenzolylethylene to dibenzolylethane, where the latter cannot be oxidized no matter how strong the oxidizing agent (Conant, 1926).

There is a certain range in which neither oxidation of luciferin nor reduction of oxyluciferin occurs. Perhaps we are justified in speaking of both apparent oxidation and apparent reduction potentials, the "apparent reduction potential" differing from the "apparent oxidation potential" (in Conant's sense, 1926) by about .5 volt.

The preceding experiments also prove that oxidation of luciferin may occur without oxygen (and without luminescence). What are the conditions for oxidation with luminescence? We know that luciferase is necessary for luminescence. Are *both* luciferase *and* oxygen necessary, or will luminescence appear in presence of luciferase alone, if oxidation of luciferin is brought about by $K_3Fe(CN)_6$? It is found that both luciferase and oxygen are necessary, for on adding potassium ferricyanide solution free of oxygen to a mixture of *luciferin and luciferase in absence of oxygen* (and of course dark) no luminescence will appear, although the luciferin will be oxidized, as is proved by the absence of light when oxygen is later admitted to the vessel.

We may suppose that the oxidation of luciferin by oxygen at the surface of colloidal particles of luciferase results in luminescence. The light is characteristic of the luciferase rather than the luciferin, since the color of the luminescence may be shown to be dependent on the kind of luciferase used and not on the luciferin (Harvey, 1917, 1924, *a*). Will any other easily oxidizable compounds luminesce when oxidized by oxygen *in presence of luciferase?* All my attempts to find such substances have failed, although I have tested many hydroxy- and aminophenols, leuco dyes, cysteine, and reduced bodies of unknown composition formed by reduction in tissue extracts of various animals, yeast and bacterial cultures.

For luminescence of *Cypridina* it is therefore necessary to have luciferin, luciferase, and free oxygen dissolved in water. It makes no difference how rapidly luciferin is oxidized by oxygen (for instance at high temperatures), luminescence never appears unless luciferase is also present, nor will luminescence appear in presence of luciferase unless oxygen is the oxidizing agent. Therefore, high reaction velocity *per se* is not a necessity for luminescence. But if luciferase and oxygen are present, then the greater the reaction velocity, the brighter will be the luminescence (Amberson, 1922).

SUMMARY.

The oxidation-reduction potential of the *Cypridina* luciferin-oxyluciferin system determined by a method of "bracketing" lies somewhere between that of anthraquinone 2-6-di Na sulfonate (E_o' at pH of 7.7 = −.22) which reduces luciferin, and quinhydrone (E_o' at pH of 7.7 = +.24), which oxidizes luciferin. Systems having an E_o' value between −.22 and +.24 volt neither reduce oxyluciferin nor oxidize luciferin. If the luciferin-oxyluciferin system were truly reversible considerable reduction and oxidation should occur between −.22 and +.24. The system appears to be an irreversible one, with both "apparent oxidation" and "apparent reduction potentials" in Conant's sense. Hydrosulfites, sulfides, $CrCl_2$, $TiCl_3$, and nascent hydrogen reduce oxyluciferin readily in absence of oxygen but without luminescence.

Luminescence only appears in water solution if luciferin is oxidized by dissolved oxygen in presence of luciferase. *Rapid* oxidation of luciferin by oxygen without luciferase or oxidation by $K_3Fe(CN)_6$ in presence of luciferase but without oxygen never gives luminescence.

BIBLIOGRAPHY.

Amberson, W. R., 1921–22, *J. Gen. Physiol.*, iv, 517.

Clark, W. M., 1925–26, *Chem. Rev.*, ii, 127.

Clark, W. M., and collaborators, 1923–26, *U. S. Pub. Health Rep.*

Conant, J. B., 1923, *J. Biol. Chem.*, lvii, 401; 1926, *Chem. Rev.*, iii, 1.

Conant, J. B., and Cutter, H. B., 1924, *J. Phys. Chem.*, xxviii, 1096.

Conant, J. B., and Fieser, L. F., 1924–25, *J. Biol. Chem.*, lxii, 595, 623.

Harvey, E. N., 1917, *Science*, xlvi, 241; 1918–19, *a*, *J. Gen. Physiol.*, i, 133; 1918–19, *b*, *J. Gen. Physiol.*, i, 269; 1920, The nature of animal light, Philadelphia and London; 1922–23, *J. Gen. Physiol.*, v, 275; 1924, *a*, *Am. J. Physiol.*, lxx, 619; 1924, *b*, *Physiol. Rev.*, iv, 639.

GALVANOTROPISM AND "REVERSAL OF INHIBITION" BY STRYCHNINE.

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I.

Definite neuromuscular effects are produced in a variety of organisms by the passage of a constant electric current. The character of the induced galvanotropic curvatures and movements has played a certain part in the development of the tropism doctrine (*cf.* Loeb, 1918), but these reactions still await quantitation—which is difficult —as well as fuller utilization for the analysis of animal movements. The interpretation of galvanotropism in metazoans turns at present upon the necessary and sufficient assumption that the current serves to excite definite groups of nerve cell bodies (presumably as determined by the axial orientation of these cells with respect to the polarity of the current—Loeb and Maxwell, 1896; Loeb, 1918; Moore, 1922–23, *b*).[1] The effects are such that among annelids, for example, the animal typically extends and lengthens when the head is toward the cathode, but shortens when the current is in reverse direction. These effects are in certain respects similar to those produced by neurophil drugs. Strychnine, for example, induces a similar and comparable elongation, whereas nicotine leads to shortening. It was considered that if the effects of such alkaloids are indeed due to selective or differential unions with particular groups of nervous elements, the result of combining the action of a substance of this type with that of the electric current should be merely an accentuation of the primary action, since the response induced involves ac-

[1] It should be noted, however, that the apparent impermeability of cells to direct current imposes certain conditions on the form which this interpretation may take.

tivity of the same nerve-muscle groups. In certain cases this turns out to be true.

The effect of strychnine is of especial interest. In the spinal cord of vertebrates it is usually supposed (Starling, 1926) that the strychnine effect is due to abolition of the inhibitory component of normal coordination, so that the inhibitory effect is transposed into an excitatory one. Coordination thus becomes impossible, for antagonistic muscles contract together, so that the resulting movement is determined by that muscle group which is the stronger. The locus of this action is commonly assumed to be at synapses (or their homologues), but until the nature of inhibition is better understood it might be assumed that the strychnine effect may really involve the elective excitation of nerve cell bodies.

Reversal of reaction by strychnine is apparent in a variety of invertebrates[3] in which the coordination of antagonistic muscle groups is requisite for normal movements. If the effect of the strychnine is primarily one depending upon the elective excitation of particular nerve cells, then a strychninized annelid, in which the longitudinal muscles are relaxed, circular muscles contracted, so that the animal is much elongated, should be caused to elongate still further when submitted to the action of a constant current with cathode at the head. The fact is that under these conditions the usual cathodic galvanotropism is reversed; the animal shortens when the head is toward the cathode, elongates when the head is toward the anode. The simplest interpretation of this result is that the reversal effect of strychnine is a true synaptic effect not explained by elective excitation of nerve cells, and the experiment therefore becomes a test of the nature of at least certain types of reversal of inhibition.

It is proposed to illustrate this point by means of tests with platyhelminths, nemerteans, and annelids.

II.

Observations on the galvanotropism of common shore nemerteans were made in glass troughs containing sea water, through which a current of about 4 volts, 0.5 milliamperes per sq. cm., was sent

[3] *Cf.* Moore, 1918–19, 1919–20; 1923–24, *a*. Crozier, 1919–20, 1922.

by means of cotton pads. The current density was varied by changing the cross-section.

Several species of *Lineus* are pronouncedly cathodic. Most of the experiments were made with *Lineus socialis;* similar results were also obtained with *L. viridis*, a species of *Tetrastemma*, and another of *Cerebrotulus*.

In creeping the body is moderately elongated, and there is frequently no evidence of peristaltic waves; sometimes a succession of such waves may be apparent. When a current is sent through the water the nemertean undergoes marked elongation if the head is toward the cathode, and creeping continues in that direction. If the current is reversed, the body promptly shortens, and the anterior end, now toward the anode, becomes notably shortened and thickened; the worm may then move (backward) toward the cathode, but soon the anterior end extends to one side and both ends of the body are then directed to the cathode. Continued creeping brings the nemertean into cathodic alignment with the current. If at first the animal is transverse to the lines of current flow, both ends are at once directed to the cathode, the body becoming bent in a U. This behavior is essentially like that of the earthworm (Moore and Kellogg, 1916; Hyman and Bellamy, 1922; Moore, 1922–23, *b*) and is shown by other annelids, *Nereis* (Hyman and Bellamy, 1922) and *Harmothoaë*, as well as by marine and fresh water planarians. If the current be sent through the nemertean dorsoventrally, again the longitudinal muscles on the cathode side contract and produce the U-posture. As in the case of the earthworm (Moore, 1922–23, *b*), pieces or regions of the body exhibit similar responses. If a portion only of the intact nemertean be suspended in the current-trough certain differences appear. With the anterior end submerged and hanging vertically, closure of the current results in cathodic orientation and elongation of the submerged part; reversal of the current produces extensive shortening, so that the immersed part is lifted out of the water. Immersion of the posterior end only shows that part orienting with the posterior tip toward the anode, and reversal leads to contraction of the longitudinal muscles.

Isolated anterior ends of *Lineus* orient cathodically and elongate in this direction, shortening and turning on reversal. A transverse

current produces the U-form. The behavior of isolated posterior parts is somewhat more complicated. Longitudinal muscles contract on the cathode side,—except occasionally just after cutting, when those on the anode side contract,—so that the typical U effect of a transverse current is produced; and the contraction of circular and dorsoventral muscles, with relaxation of longitudinals, leads to the usual elongation when the posterior end is toward the anode, which is changed to a shortening and thickening of the piece when the current is reversed. But peristaltic waves are commonly initiated at whichever end of the body happens to be directed toward the anode; and the piece creeps toward the anode whether the creeping be posteriorly or anteriorly directed; in a number of preparations it was clear that when in a U, with the two ends toward the cathode, peristaltic waves began at the anodic bend and moved, more or less alternately, to either end. Elongation of the fragment, however, occurs only when the posterior end is toward the anode, and reversal of the current produces the usual shortening and thickening; creeping with the anterior toward the anode is very slow, with faint, well spaced peristaltic waves and some wrinkling due to local circular muscle contraction. Occasionally, if antero-posterior peristalsis has been initiated it persists on reversal of the current, so that the piece creeps to the cathode. The behavior of pieces cut from the mid-region of the body, and thus with two cut surfaces, is similar. These, and the other isolated parts, were usually kept for about 24 hours before being tested.

The long proboscis of *Lineus* is not easily discharged.[3] Since the proboscis contains circular and longitudinal muscles it was desired to test its galvanotropism apart from the control of the nervous system in the body of the worm. The extracted proboscis shortens when its distal end is toward the cathode. Short fragments show longitudinal contraction on the cathode side. In thus paralleling the behavior of the whole body the proboscis is similar to the isolated tentacles of *Polyorchis* (Bancroft, 1904). It is an interesting question, however, as to the possibility of independent galvanotropic

[3] The proboscis of *Tetrastemma* was occasionally found to be everted when the anterior end was turned toward the anode, and retracted when this end completed its cathodic orientation.

behavior of the parts of an animal. An illustration is afforded by the ceratia of eolids. *Æolida diversa* is cathodic, as are other nudibranchs which have been tested. *Æolida*, and *Dendronotus arboreacens*, elongate when the anterior end is toward the cathode; the tentacles and "rhinophores" bend to the cathode, but the ceratia (all but the most anterior four or six, in *Æolida*) bend to the anode. Reversal of the current causes retraction of oral and dorsal tentacles, shortening of the body, and the ceratia on the posterior part of the body stand out sharply and turn toward the anode. If the current is transverse, the animal shows extension of the ceratia only on the anode side. When autotomized posterior ceratia are studied it is found that they shorten when the distal tip is toward the cathode, elongate when the base is at the cathode; a transverse current produces longitudinal contraction on the anode side, so that both ends point to the anode. This effect cannot be due merely to the absence of the central ganglia because both anterior and posterior halves of transversely bisected individuals are found to creep and to orient cathodically. A further illustration of this sort of difference between the behavior of the body as a whole and of an appendage is found in *Echiurus*.

The galvanotropism of *Echiurus chrysacanthophorus* was of especial interest for the experiments in view, because the orientation is anodic. A current sent transversely through the body leads to longitudinal contraction on the anode side, the body being thrown into a U. As the oral end turns to the anode, the circular muscles contract and this end elongates. Both normal and small regenerating proboscides, however, extend toward the cathode. With the body lengthwise in the current elongation is shown, particularly at the oral end, when the oral end is toward the anode; the proboscis is turned back toward the cathode. Peristaltic waves run from oral to aboral pole. When the current is reversed, the aboral end, now anodic, elongates markedly, and the oral end swings around so as to be directed to the anode. In some cases, locomotion being difficult on the smooth glass bottom of the trough, the animal progresses toward the cathode. Attention was chiefly given, however, to the interplay of circular and longitudinal muscles as the animal lay lengthwise to the current. Here it is obvious that longitudinal contraction is determined when the oral end is toward the cathode, longi-

tudinal elongation and circular contraction when the oral end points to the anode. The isolated proboscis, however, shortens and thickens, its lateral edges and the distal end curling together, when the cathode is at the base of the organ; current reversal produces uncurling and longitudinal extension. Thus the proboscis may be considered intrinsically cathodic in its orientation, in contrast to the behavior of the body of the gephyrean.

The foregoing refers to the orientation of *Echiurus* lying on the bottom of a glass dish. At night, however, as described by Wilson (1899–1900), the worm can swim in an aquarium by means of vigorous peristaltic movements of the body wall; this performance is very striking in view of the mud-dwelling habit of the worm. When actively swimming *Echiurus* orients and swims to the cathode. Swimming movements were excited by repeated rapid reversals of the galvanic current, and orientation was then cathodic. Reversal of normally cathodic galvanotropism has been noticed in *Nereis* long confined to the laboratory (Hyman and Bellamy, 1922), but this is not the explanation of the behavior of *Echiurus*.

III.

The usual effect of strychnine upon flatworms and annelids is to produce (Knowlton and Moore, 1917; Moore, 1918–19) an enhanced excitability, and a reversal of the usual reaction to a local touch, for example, so that the contraction of longitudinal muscles is replaced by extension and the contraction of their antagonists. It is commonly observed that the gross effect of nicotine is to produce a shortening, thickening, or ventral flexure of the body of worms and other invertebrates (*cf.* Moore, 1919–20; Crozier, 1919–20; Crozier and Federighi, 1924–25). In the flatworms, nemerteans, and gephyrean used in the present experiments no unusual effects of strychnine or of nicotine were obtained, but it is necessary to record the results briefly, for the interpretation of the tests with the galvanic current.

Lineus in aquaria tends to collect in dark corners, but its negative phototropism is not pronounced except when first collected. The initial noticeable effect of treatment with strychnine sulfate (1:50,000) is the marked enhancement of this phototropism. Later a more or less stationary transverse enlargement of the body is apparent, at the

level of the mouth; the lips are widely separated and the pharynx open. This is succeeded by the addition of more "standing waves", posterior to the mouth level; soon the whole body becomes much elongated. With higher concentrations the anterior half of the animal ultimately swells due to relaxation of the circular muscles as well as of the longitudinals, and the whole body is quite flabby. In solutions as concentrated as 1:2,000 the posterior region is very much attenuated, and the constriction of circular muscles pinches the body into fragments. Before this happens the body may be thrown into a coil, and in this the *dorsal* surface is always contracted, so that the animal may be said to be opisthotonic.

In the stage of excessive elongation, which with proper concentration of the drug lasts for some hours, creeping is entirely normal, but the reactions to tactile stimulation are reversed. A touch with a needle on the dorsal mid-surface of a normal *Lineus* leads to quick circular constriction at that level, coupled with longitudinal muscle contraction immediately anterior and then posterior; the two contractions of the longitudinal musculature run as waves in either direction; the circular constriction at the point touched persists for a time which varies with the severity of the excitation. If the animal be lifted from the bottom with a needle, it shortens, thickens, flattens; then the anterior end is extended and the circular constriction there begun progresses posteriorly.

A strychninized *Lineus* exhibits a stage in which the longitudinal shortening occurs anterior to the level of a touch, but the posterior part shows only a prompt lengthening. Then a little later it is seen that a gentle touch produces, not local circular constriction with attendant longitudinal contractions, but a local shortening *after* a local extension. The full development of this phase of the strychnine effect finds the animal responding to a touch by a violent contraction of the circular muscles, so that the whole body is enormously thinned and elongated. This reversal is also shown when the anterior end, with its ganglia, has been amputated, and indeed by short posterior pieces. At no time, however, does the animal spontaneously creep backward.

The two points which are of interest in the present connection are (1) the general elongation of the animal by strychnine, comparable

to the galvanic effect with cathode at the anterior end, and (2) the reversal in the action of circular and longitudinal muscle groups following local stimulation.

There is one pertinent mode of response which does not seem to be affected by strychnine, at least until creeping is no longer possible. According to Moore (1923–24, *b*) the homostrophic reflex which tends to maintain the pursuit of a straight path in annelids (Morgulis, 1910; Moore, 1922–23) and in some arthropods (Crozier and Moore, 1922–23; Crozier, 1923–24) is not exhibited by *Cerebratulus*. The interest of this fact comes in connection with the suggestion (Moore, 1923–24, *b*) that the reflex excited by a lateral curvature of the body and which results in the anterior part being brought into alignment with the tail, might in some way be connected with segmental organization, since it is not seen in *Cerebratulus*, in planarians, or in the slug *Limax maximus*. But in a variety of the smaller nemerteans it is easily shown that precisely this homostrophic orientation of the anterior end is an important feature in directed creeping. Within a region extending back some 2 cm. from the anterior end typical homostrophic response is obtained in forward creeping, if the anterior end has been bent sharply to one side; slighter lateral displacements are effective nearer the anterior end. In backward creeping the posterior end continues to travel in the direction in which it is put, but the homostrophic adjustment of the anterior end is evident when this part of the animal travels backward through an impressed bend; the homostrophic orientation is therefore not dependent upon the direction of progression, but upon a central nervous state connected with the creeping act. "Beheaded" worms fail to show this behavior. The homostrophy of normal animals is accentuated by treatment with nicotine, but continues absent at other levels of the body.

IV.

When *Lineus* in the stage of strychninization characterized by elongation of the body and by reversal of the usual reaction to touch are subjected to the action of the galvanic current it is found that they *shorten* when the head is turned toward the cathode, *elongate* further if the anterior end is toward the anode, and that if placed

transversely both ends of the body are turned to the anode. There is thus complete reversal of the usual galvanotropic behavior.

No reversal of galvanotropism could be secured by varying the current density. But at a slightly earlier stage in the strychnine action it is found that the longitudinal muscles on the cathode side of an animal transverse to the current will be contracted, as normally, but that when the anterior end is thus turned to the cathode the whole longitudinal musculature contracts and the worm shortens. To bring this about it is sufficient merely to bend the anterior end at the level of the mouth, so that the "head" is toward the cathode, the rest of the worm being parallel to the current lines, with posterior end to the cathode. Cases are found, also, in which the body is bent in a U with both ends toward the cathode, but with the longitudinal muscles contracted on the anterior half of the body.

These observations speak for the localization of the mechanism of strychnine reversal at the anterior end of the body. When the anterior part is amputated, at a level slightly posterior to the mouth, the worm shortens when the posterior end is at the cathode, elongates when the current is reversed. This proves that the strychnine reversal in the intact *Lineus* is due to an effect upon structures at the anterior end of the body, and it is natural to conclude that the cerebral ganglion is the essential locus. The isolated "heads" elongate with anterior tip toward anode, shorten on reversal. The "head" region so defined agrees with that evidenced by the homostrophic responses.

Isolated posterior halves are found to orient cathodically, although the end toward the cathode is broader than the posterior end, toward the anode. When the current is reversed the circular muscles at first contract, producing anodic elongation, but the anterior end turns toward the cathode.

V.

The galvanotropism of *Echiurus* supplies a complementary case. Treatment with strychnine (about 1:20,000, or less) causes the body to become more cylindrical, the circular muscles relatively more contracted; the proboscis is quite extended. Placed transversely to the current, both ends are bent toward the *cathode*, and the proboscis

extended in this direction. Reversal of the current causes first the proboscis, then the oral end, then, less vigorously, the aboral end, to be swung toward the new cathode. In line with the current flow, the body extends when the oral end is cathodic, shortens when the oral end is anodic; in the first case the proboscis is extended, in the second bent back toward the cathode.

The galvanotropic behavior of the body muscles is therefore reversed by strychnine. But the conduct of the proboscis is unchanged. The isolated proboscis presents very much the appearance of a non-strychninized one, save that the distal end is kept more curled together and that its reactions are slower. Its behavior toward the galvanic current is not altered by the strychnine. The sort of independence of the central nervous system exemplified by the proboscis of *Echiurus* (and by the ceratia of *Æolida*) it is permissible to correlate with the fact that these organs are readily lost by autotomy.

VI.

These experiments began with the thought that the contraction of circular muscles, relaxation of longitudinals, characteristic both of the strychnine effect and of cathodal galvanotropism in *Lineus*, involve the activity of the same neuromuscular elements. The result showed that the typical effect of strychnine is not enhanced by the corresponding effect of the current, but that on the contrary the usual galvanotropic orientation suffers reversal under strychnine. The obvious conclusion is that whether or no strychnine "excites" by stimulation of nerve cell bodies, it must also, or perhaps primarily, have some other action which produces a true "reversal of inhibition"; and it is difficult to avoid the assumption that the locus of this reversal is in synapses (or their homologues). The results of removing the "brain" of *Lineus* strengthen this assumption, as does also the absence of the reversal in the proboscis of *Echiurus*. The fact that both the cathodal galvanotropism of *Lineus* and the anodal galvanotropism of *Echiurus* may be reversed by strychnine disposes of certain interpretations which might conceivably be put upon the behavior of one of these forms by itself.

A further test of this conception of the place of action of strych-

nine in effecting reversal of reaction is given by experiments with nicotine. Most of these experiments were made with several genera of marine platyhelminths. Exposure to dilute nicotine solution in time causes the longitudinal muscles of these worms to contract, the circular and transverse fibers to relax, so that the body is short and swoolen; the ventral longitudinal muscles are so contracted as to arch the body dorsally. If the galvanic current, presumed to act upon nerve cell bodies, should produce normally a similar effect, a common locus for the action of current and of nicotine would have to be assumed. Nicotine does not reverse the cathodic galvanotropism of planarians, nor of *Lineus*. But, in nicotine solution, even before the characteristic picture of nicotinization has begun to appear, the shortening, flattening, and arching of the body later produced by the alkaloid is clearly brought out during the flow of the galvanic current. The animals orient and move toward the cathode, but the body is broad and swollen, especially at the anode.

SUMMARY.

The cathodically galvanotropic orientation of nemerteans, *Lineus*, and the anodic orientation of the gephyrean *Echiurus*, are reversed by the action of strychnine under conditions such that the typical "reversal of inhibition" induced by this substance is apparent. Nicotine does not give this result. Since it is necessary to assume that the strychnine effect is due to action upon the central ganglia, and since the galvanotropic effect depends upon action of the current on nerve cell bodies of the central ganglia, it must be assumed that the locus of reversal by strychnine is not perikaryal, but presumably synaptic.

CITATIONS.

Bancroft, F. W., 1904, *J. Exp. Zool.*, i, 289.

Crozier, W. J., 1919–20, *J. Gen. Physiol.*, ii, 627; 1922, *Biol. Bull.*, xliii, 239; 1923–24, *J. Gen. Physiol.*, vi, 531.

Crozier, W. J., and Federighi, H., 1924–25, *J. Gen. Physiol.*, vii, 221.

Crozier, W. J., and Moore, A. R., 1922–23, *J. Gen. Physiol.*, v, 597.

Hyman, L. H., and Bellamy, A. W., 1922, *Biol. Bull.*, xliii, 313.

Knowlton, F. P., and Moore, A. R., 1917, *Am. J. Physiol.*, xliv, 490.

Loeb, J., 1918, Forced movements, tropisms, and animal conduct, Monographs on experimental biology, Philadelphia.

Loeb, J., and Maxwell, S. S., 1896, *Arch. ges. Physiol.*, lxiii, 121.

Moore, A. R., 1918–19, *J. Gen. Physiol.*, i, 97; 1919–20, *J. Gen. Physiol.*, ii, 201; 1922–23, *a*, *J. Gen. Physiol.*, v, 327, 451; 1922–23, *b*, *J. Gen. Physiol.*, v, 453; 1923–24, *a*, *J. Gen. Physiol.*, vi, 73, 281; 1923–24, *b*, *Proc. Soc. Exp. Biol. and Med.*, xxi, 365.

Moore, A R., and Kellogg, F. M., 1916, *Biol. Bull.*, xxx, 131.

Morgulis, S., 1910, *J. Comp. Neurol. and Psychol.*, xx, 616.

Starling, 1926, Human physiology, Philadelphia.

Wilson, C. B., 1899–1900, *Biol. Bull.*, i, 163.

PHOTOTROPISM IN YOUNG RATS.

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I.

A good deal has been written as to the limits of tropistic interpretation, particularly with regard to the behavior of higher animals such as hymenopterans and mammals. It seems to us that for any one who has seriously concerned himself with the analysis of animal conduct these discussions are for the most part extremely unsatisfactory. The study of the behavior of vertebrates has probably suffered a good deal from historically derived preoccupation with psychological questions. Perhaps the chief difficulty has been the absence of experiments likely to produce clear-cut and fruitful ideas. We are aware that to many the mere mention of "clear-cut ideas" in such connection is unpleasant. What we propose, however, is a system of analysis deliberately based upon a notion which to some would unquestionably appear a pernicious simplification. We have sought to discover well defined instances of tropistic conduct in mammals. These are of intrinsic interest. But their ultimate utility for our purpose consists in the fact that these tropisms, if found, may be brought into conflict, and the resolution of such conflicts studied under varied conditions (*cf.* Crozier, 1923–24, *a*, *b;* Crozier and Federighi, 1924–25, *a;* Crozier and Stier, 1926–27). It seems to us that the procedure of which this outline is a very bald statement should make possible a method of approach for the investigation of central nervous states otherwise inaccessible to measurement. It is a method, moreover, which frankly recognizes moment-to-moment variability in conduct, but which seeks to obtain quantitative evidence as to the nature of this variability and its physical basis.

It is necessary to find organisms capable of providing experimental results of the type required. For several reasons we have employed

young rats and mice. Suitable tests have shown that they exhibit modes of behavior adapted for our purpose. And enough is known about them genetically to encourage the hope that it may be possible to utilize hereditary genes for the production of desired modifications of structural and other features.

The stereotropic behavior of these mammals has been described in a previous paper (Crozier and Pincus, 1926–27, *a*). We propose now to present proof of truly heliotropic conduct in young rats. It happens that the experiments seem not without significance for certain aspects of the general theory of phototropism. Beyond this, the occurrence of positive stereotropism and of negative phototropism during creeping makes it possible to bring these individually definite and predictable modes of response into conflict, in such a way that the animal must be guided by one form of response to the exclusion of the other, and to observe the manner in which the resolution of such conflicts may be modified. The nature of the results under these conditions will be discussed on another occasion.

II.

Conceived as a forced movement of the organism as a whole, tropistic conduct requires the presence of a preexisting mechanism, sometimes referred to as a "behavior pattern" (Child, 1924)—although this phrase may not in itself be particularly illuminating—so that the mode of response is a direct expression of the way in which the organism is constructed; and indeed there exist types of phototropic response, for example, which need not be interpreted in terms of adaptive requirements (Crozier and Arey, 1919–20). When the behavior of higher animals, such as man, is examined, attempts have been made to refer to the category of tropisms such phenomena as the guidance of movement by ideas (Royce, 1903) or by "memory images" (Loeb, 1918). Before analysis can make more than figurative headway in this direction it is necessary to discover if any simple and recognizable tropistic movements occur in mammals. The case of stereotropism has recently been discussed (Crozier and Pincus, 1926–27, *a*). The proof of geotropism is even more illuminating (Crozier and Pincus, 1926–27, *b*). To test for the presence of phototropism certain difficulties must be obviated. Complex image-forming or eidoscopic eyes,

with pronounced central nervous connections, obscure potential tropistic response (Rádl, 1903; Parker, 1903; Parker, 1922, *b*). Thus the seeming positive phototropism of the young loggerhead turtle moving toward the sea (Hooker, 1911) is apparently the result merely of the retinal image of an unbroken horizon (Parker, 1922, *a*). Corresponding phenomena complicate the phototropism of imaginal insects (Rádl, 1906; Parker, 1903; Crozier and Federighi, 1924–25, *b*).

The eyes of nestling rats of the strain used do not open until the 14th day after birth. For some days before the eyelids open these animals respond to light. But during this time the eye must be regarded as a "direction eye" (euthyoscopic), not as an image-forming organ. Thus the opportunity is given to study the reactions induced by illumination, apart from the rôle of images, and apart from the effects of memory traces. The experiments may at once be controlled by opening the eyelids to discover the influence of retinal images of illuminated fields. The result shows that the closed lids provide a natural screen which precludes the formation of definite images, but which permits photic excitation.

Young rats of dark-eyed, black hooded stock were principally used in these experiments. The hooding factor produces a narrow band of dark hairs along the mid-dorsal line, which greatly facilitates the taking of graphical records of individual movements. While the eyelids are still closed the rats invariably move away from a source of light, with a promptness and precision rivalling that of a blow-fly larva. This is best seen between the ages 8 and 14 days; before the 8th day creeping is too uncertain to permit very definite orientation.

III.

One means of testing the phototropic nature of this response is to determine the character of the reaction when the animal is influenced by two sources of light. The lights used in these experiments were opposed at 180°. Young rats which start creeping on a line midway between two lights of equal intensity orient themselves in a direction perpendicular to the line joining the two lights (*cf.* Loeb, 1905; Patten, 1914). If the lights are of unequal intensity the path of oriented creeping is deflected toward the weaker light.

A black cloth, fastened to the observation table, permitted the

animals to obtain a firm creeping grip. A system of circular and radial coordinates was marked in white upon the cloth, and allowed the changing position of the animal to be charted upon similarly marked record sheets. Creeping was begun near the center of the observation table, and the path was recorded until the outer marked circle had been reached. Only such complete trails were studied, the few instances being ignored in which, after very brief creeping, the animal squatted down on the cloth and refused to move. Each rat was "run" repeatedly, first with one side toward the stronger light, then the other. The intensities of the two lights were measured

TABLE I.

Angles of orientation toward the weaker light, I_1 and I_2 opposed at 180°; H is the calculated effective inclination of the photoreceptive surfaces (see text).

I_1	I_2	θ	H/2
foot candles	*foot candles*		
8.0	8.0	0.19° ± 0.01	
8.0	7.0	5.98° ± 1.2	24.4°
8.6	7.0	15.8° ± 1.4	19.9°
8.6	5.7	22.4° ± 1.1	28.0°
8.6	4.6	27.9° ± 1.8	29.5°
8.6	3.9	32.2° ± 2.0	35.2°
8.2	3.4	31.1° ± 2.5	38.7°
9.5	3.1	41.0° ± 3.0	30.3°
10.3	2.9	44.3° ± 2.0	30.5°
12.0	2.67	47.4° ± 2.1	30.3°
14.0	2.65	52.0° ± 3.3	25.9°

directly in the zone of creeping. Nitrogen-filled bulbs served as sources. The angle of orientation was measured from the charted record of each orientation test.

The results of different series of experiments are collected in Table I. The consistency of the measurements of the angles of orientation, especially as seen in the small probable errors of the means, is surprisingly good.

Orientation by lights opposed at 180° should be definable (Crozier, 1926–27) by the equation

$$\tan \theta = \left(\frac{I_1 + I_2}{I_1 - I_2}\right)\left(\cot \frac{H}{2}\right) \qquad (1)$$

where θ is the angle between the line of orientation and the normal to the path of the light beams, I_1 and I_2 are the intensities of the lights ($I_1 > I_2$), and H is the average angle between the photoreceptive surfaces. It has been pointed out (Crozier, 1926–27) that this angle, H, should increase if orientation becomes more precise with increasing total acting light intensity; or, on the other hand, decrease if predominantly influenced by photokinetic movements; in either case H should be very nearly proportional to $\log I_1 I_2$. In this way the variation of $H/2$ with $\log I_1 I_2$ gives a test of the formula,—especially since cases are available in which H does not appreciably vary at all, and for which equation (1) holds with all desirable precision. For the rats, H decreases almost linearly as $\log I_1 I_2$ increases (Crozier, 1926-27). An approximate graphical illustration of the relations between θ, I_1, and I_2 may be given (Fig. 1) by using the empirical relation

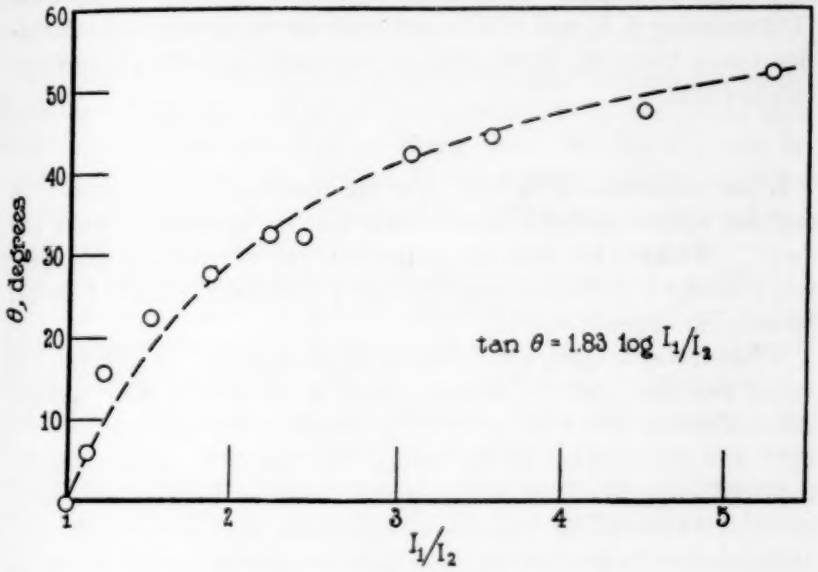

FIG. 1. The empirical formula $\tan \theta = K \log I_1/I_2$ gives an approximate description of the variation in angle of orientation as dependent upon I_1 and I_2, and may be used in order to obtain a graphical representation of the results.

$$\tan \theta = K \log I_1/I_2 \qquad (2)$$

obtained by M. M. Moore (1923–24) for such cases. It has been shown (Crozier, 1926–27) why, for ordinary ranges of intensities, this may give a very fair expression of the facts.

When I_1 and I_2 are equal, the path of orientation should be normal to the line joining the sources of illumination. The experimental finding of a mean deviation of only $-0.2° \pm 0.01$ is probably within the error of the method of recording. Beyond this, the way in which θ increases as the ratio I_1/I_2 is altered shows that the orientation fully obeys the primary requirement of phototropism, for the equation (1) connecting θ, I_1, and I_2 is based simply on the theory that orientation ceases when the illumination of bilaterally disposed photoreceptors is equal.

IV.

It has sometimes been held that the results of experiments with opposed sources of light mean merely that the organism "seeks the dark". With the rat there is a rather neat way of removing this opinion, although it is obvious that the results contained in Table I should be sufficient demolition of it.

When the eyes open, a different situation results. In general, the young rats then seek the darkest region in the field of view, usually the shadows on either side of the lamp house. Older animals face the light, and creep toward the darkness at one side of it. If a young rat is placed facing the light, and at a point midway between two symmetrical zones of shade, it moves *away* from the light, in a straight line. If, however, it be placed nearer one shadow than the other it moves at once to the nearer, even moving almost directly *toward* the light to do so. If put at some distance from a lamp (say, 6 feet from a 100 watt bulb), it usually turns away from the light toward the recesses of the dark room. There seems to be a zone in which the tendency to turn away from the light and the stimulus leading to creeping toward the dark corners at the sides of the lamp house are about equalized. Thus even with the eyes opened there is still some evidence of negative phototropism, but it is clear that there is superimposed upon its simpler manifestation the positively orienting influence of darkened areas contrasting with a brighter one in the visual field (*cf.* also the results of learning tests with rats; Yerkes, 1907; Watson, 1914).

The type of response determined by the visual effect of darkness is obtained in rats kept in the ordinary illumination of the laboratory, or in those continually maintained in darkness until after the eyes have opened. It is secured at once if the eyelids are opened by operation before their normal time. This makes it possible to show directly that if the animal were "seeking the dark", in the sense in which this expression may be used with reference to vision, it would scarcely move in the way recorded in Table I.

V.

Another way of testing the occurrence of phototropism has to do with the examination of circus movements. With the young rats there are three possible ways of doing this. (*a*) One eye may be opened, at the age of, say, 12 days, the other eye remaining closed; or (*b*) one of the two unopened eyes may be covered by an opaque screen; or (*c*) one eye may be extracted.

In the first case, (*a*), care must be taken to prevent the possibility of image formation in the opened eye, by presenting a uniform visual field. When this is done it appears that the primitive phototropic effect, already noted as persisting in connection with the recently opened eye, brings it about that the rat circles slightly ***toward the unopened eye.*** This would result if stimulation of the opened eye were merely more intense, which is pretty certainly the case. These tests were made by illuminating prepared rats by a ring of electric bulbs suspended from a large circular metal frame, within which the rat crept. The lamps were brought within 3 feet of the creeping surface. This method is necessary to avoid shading of one eye by the rat's head, as when a single point source is used. With ***high*** total intensities of light (1000 m.c.) no definite circling to either side is obtainable.

The second method, (*b*), has disadvantages, because it is difficult to attach an adequate screen which the animal will ignore and which is small enough not to interfere with the vibrissæ.

The third procedure, (*c*), results in very clear-cut exhibitions of maintained circling toward the blind side. The amount of turning depends, as in *Limax* (Crozier and Federighi, 1924, *a*) upon the speed of creeping. With high intensities of illumination the rat squats more or less at one point and pivots about the strongly flexed hind limb of

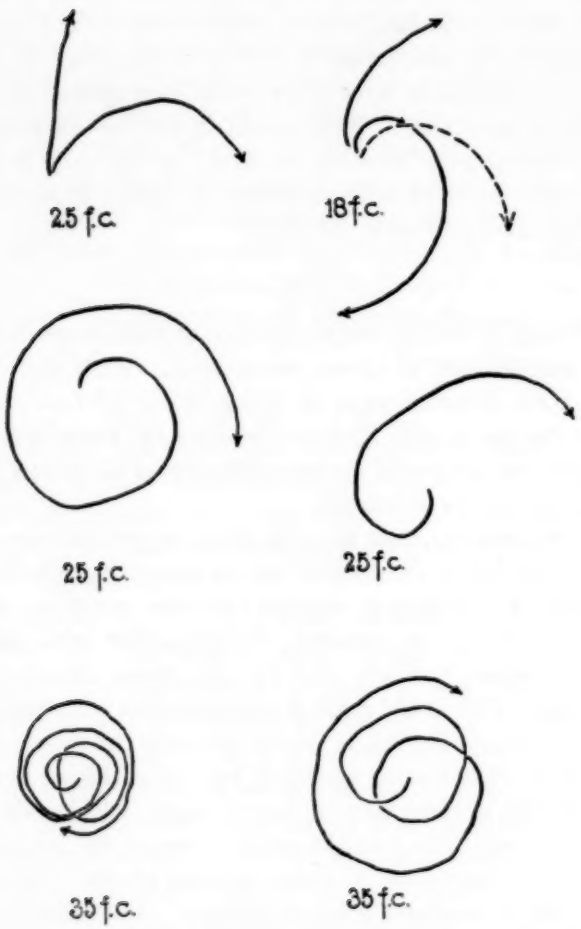

FIG. 2. Circus movement trails of young rats with one eye (the right) removed, the eyelids of the other not yet parted. Illumination from a single source, vertically above the center of the creeping stage. Intensities of the lights (foot candles) measured photometrically at the creeping stage. Scale 1:10. These trails are typical. The amplitude of the orientation movement increases with the intensity of the light. This is in part due to the fact that the animal creeps little but turns constantly. The records were obtained by following the positions of a marked spot at shoulder level on the animal's back, as seen against a system of coordinates in white lines upon a black cloth covering the creeping stage.

the blind side. More creeping movement is evident with lower intensities of light. Several typical trails of circling progression are given in Fig. 2, and fairly illustrate the type of behavior constantly observed. The postures of the legs are strikingly influenced by the light. Extensor tonus is enhanced on the photoreceptive side, and most markedly in the anterior limb; the legs of the other, blind, side are flexed, the hind leg much more so. The turning movement is brought about through the pushing effect of the extended legs, coupled with protraction by those of the blind side. The tonic effect of the

FIG. 3. The orienting posture of a rat, left eye functional but as yet unopened, right eye removed, illuminated from above.

light (Fig. 3) is as definite here as with insects (Garrey, 1918–19; Crozier and Federighi, 1924–25, *a*). Under continuous exposure the circle of turning broadens into a spiral, due to light adaptation. We may point out that this gives promise of providing a method for the quantitative treatment of light adaptation.

VI.

The result of these tests is to show that the quantitative criteria supplied (1) by the action of opposed illuminations and (2) by the requirement of circus movements, agree in demonstrating the elementary tropistic nature of the movements of the rat as influenced by

light. Upon this basic phototropism the image-forming capacity of the opened eye imposes certain modifications and restrictions. If one desires to employ quantitatively predictable behavior as an index instrument for analysis of conduct, it is necessary to choose experimental conditions such as permit the organism to display its capacities as a machine. The phototropism of the rat may thus be studied if the effect of visual images is avoided; and if young rats are employed, before the opening of the eyelids, there is not even a possibility of the memory of such images.

VII.

SUMMARY.

Before the eyelids have opened young rats are negatively heliotropic. They behave very much as does the larva of the blow-fly. The angle of orientation by lights opposed at 180° may be calculated by an equation based upon the elementary requirement of phototropism, namely that orientation is attained when the illumination of bilaterally disposed photoreceptors is equal. The precision of orientation decreases very nearly in proportion to the sum of the logarithms of the acting light intensities, due to photokinetic head movements. When the eyelids are opened, the rats move toward a darkened place in the field of vision, usually toward the shaded region immediately to one side of the lamp house. Therefore, when heliotropic, the rat is not "seeking the dark". The phototropism of these animals may be brought into conflict with their pronounced stereotropism, and the resolution of such conflicts may perhaps be utilized for the investigation of central nervous states.

CITATIONS.

Child, C. M., 1924, Physiological foundations of behavior, New York.

Crozier, W. J., 1923–24, *a*, *J. Gen. Physiol.*, vi, 531; 1923–24, *b*, *J. Gen. Physiol.*, vi, 647; 1926–27, viii, 671.

Crozier, W. J., and Arey, L. B., 1919–20, *J. Gen. Physiol.*, ii, 107.

Crozier, W. J., and Federighi, H., 1924–25, *a*, *J. Gen. Physiol.*, vii, 151; 1924–25, *b*, *J. Gen. Physiol.*, vii, 217.

Crozier, W. J., and Pincus, G., 1926–27, *a*, *J. Gen. Physiol.*, x, 195; 1926–27, *b*, *J. Gen. Physiol.*, x, 257.

Crozier, W. J., and Stier, T. J. B., 1926–27, *J. Gen. Physiol.*, x (to be published).

Garrey, W. E., 1918–19, *J. Gen. Physiol.*, i, 101.

Hooker, D., 1911, *Carnegie Institution Washington, Pub. No. 132, Papers Tortuga Laboratory*, iii, 69.

Loeb, J., 1905, Studies in general physiology, Chicago; 1918, Forced movements, tropisms, and animal conduct, Philadelphia and London.

Moore, M. M., 1923–24, *J. Gen. Physiol.*, vi, 393.

Parker, G. H., 1903, *Mark Anniv. Vol.*, 453; 1922, *a*, *J. Exp. Zool.*, xxxvi, 323; 1922, *b*, *Proc. Am. Phil. Soc.*, lxi, 107.

Patten, B. M., 1914, *J. Exp. Zool.*, xvii, 213.

Rádl, E., 1903, Untersuchungen über den Phototropismus der Tiere, Leipsic; 1906, *Biol. Centr.*, xxvi, 677.

Royce, J., 1903, Outlines of psychology, New York and London.

Watson, J. B., 1914, Behavior, New York.

Yerkes, R. M., 1907, The dancing mouse, New York.

ON THE EQUILIBRATION OF GEOTROPIC AND PHOTOTROPIC EXCITATIONS IN THE RAT.

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I.

Analysis of the phototropic movements of young rats (Crozier, 1926–27; Crozier and Pincus, 1926, 1926–27, *b*) has justified the assumption that in the absence of image formation, insured by employing animals with eyelids still unopened, these mammals behave as phototropic machines, with no detectable intervention of a central nervous factor.[1] The young rat, in a field of light, orients until the excitations on its two sides are equal; over the range of intensities used, excitation is proportional to the logarithm of the intensity of the light. Geotropic orientation of these animals, also, is proportional to the logarithm of the active gravitational pull, although the reason for this particular relationship (Crozier and Pincus, 1926, 1926–27, *a;* Pincus, 1926–27) is probably not the same as in the case of phototropic excitation.

These investigations began with the double objective of discovering the limits of tropistic analysis as it might be applied to the conduct of mammals, and of utilizing the results for the study of central functions (Crozier, 1926–27). With reference to the latter purpose it may be pointed out that there are at least two general methods possible to pursue. One of these consists in the opposition of tropistic modes of response in such ways as to permit quantitative evaluation of the resolution or equilibration of the imposed conflicts in behavior (Crozier, 1926–27; Crozier and Pincus, 1926). The conduct of the young rats in a compound field of excitation involving opposed phototropic and geotropic responses provides an elementary situation amenable to precisely this sort of treatment.

[1] Aside from that involved in photokinetic movements of the head, which is itself amenable to quantitative treatment in the same terms (Crozier, 1926–27).

The simplicity of the actual result gives the best possible proof of the adequacy of the initial assumptions with regard to the mathematical representation of the phototropic and the geotropic excitations. It also constitutes a second-order proof that in the resolution of such conflicts the rat is behaving as a machine. There are several ways in which phototropic stimulation might be conceived to modify the effectiveness of simultaneous geotropic excitation: (*a*) the constants in the equation for the geotropic effect might be modified, or its threshold altered; or (*b*) the whole form of the equation might be changed, either at once or as a function of the time of exposure to the light. The possibility first mentioned (*a*) could be dealt with very simply, and obviously could give a means for the quantitative expression such as we seek for a "central factor." The second possibility might be expected if complex psychic processes are implicated, but change with time cannot be simply tested for, because of photic adaptation. The net result of these considerations is, that if from the respective known expressions for the phototropic and the geotropic effects it is possible to predict the mathematical form of the balance between phototropic and geotropic excitations there will remain no need nor indeed any room for a psyche in this particular case. Since this is the actual result, it will be noticed that the initial assumptions of the tropism doctrine are doubly confirmed. So far as we are aware this is the first case in which just this sort of analysis has been possible, and the fact that it has been carried out with a mammal makes it all the more interesting.

II.

The rats used in previous analysis of phototropism and of geotropism were of the same genetic history as those employed for the present experiments, and in this respect the results are strictly comparable. The phototropic conduct has been treated as depending upon the fact that the excitation is proportional to log I. The geotropic response upon an inclined plane is such that the angle of upward orientation on this plane is proportional to log sin α, where α is the inclination of the creeping plane to the horizontal. If the arrangement be made such that the intensity of light is found at each of a number of

values of α which is just sufficient to suppress geotropic orientation, we should expect that

$$\log I = K \log \sin \alpha + C,$$

where K and C are constants. This could be the result only if the presence of the light failed to affect the form of the connection between geotropic excitation and response. The actual result should therefore afford a criterion for the evaluation of the kind of central nervous adjustment eventuating from the competition of the two forms of stimulation. The significance of the constant C cannot be directly determined, for we do not yet know the magnitude of the corresponding quantity in the equation for phototropic effect; but, as we shall point out subsequently, it may be estimated indirectly.

Two series of experiments were made according to the following procedure. The rats were dark-eyed, black hooded, 13 days after birth. In Series 1, three individuals were employed; in Series 2, 4,— litter mates in each case. Tests were made upon a fine-meshed wire screen stretched upon a large square platform which could be inclined at known angles to the horizontal. This creeping stage was at one end of an extended arm carrying the light source, the latter adjustable as to distance from the stage. The dark-adapted rats were placed upon the stage, one at a time, and oriented upward toward the light until the light intensity became such that the animal was forced to creep in a horizontal path. It may be assumed that at this point the upward orienting tendency is exactly counteracted. The intensities were measured photometrically, not computed. The total exposure to the light was kept at 1 minute, with at least 10 minutes in darkness between successive tests. By repeated trials the exact distance was found at which horizontal creeping was enforced; the tests were repeated five times at each inclination, for each individual.

III.

The results of the first series of tests, when $\log I$ was plotted against $\log \sin \alpha$, gave I proportional to $(\sin \alpha)^K$, with $K = 2.475$. The second series of measurements, treated separately, gave $K = 2.476$.

This degree of concordance is probably fortuitous, but we would stress the point that the individuals concerned in the two sets of determinations were unusually similar from the standpoint of age and genetic

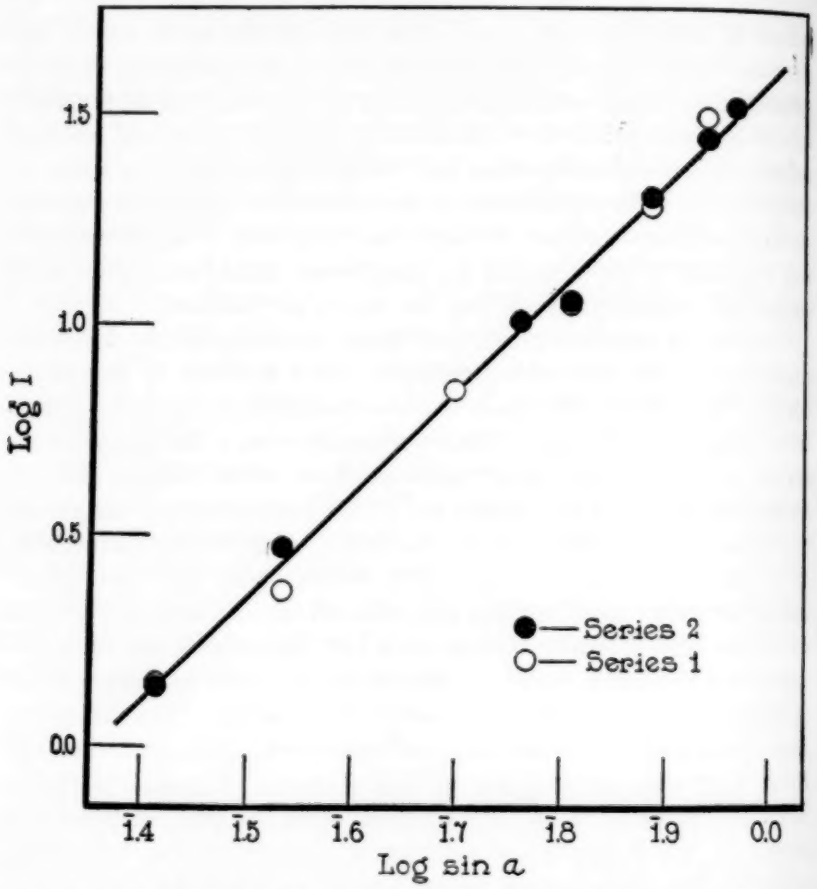

FIG. 1. The intensity of light required to inhibit geotropic orientation of young rats varies with the inclination of the creeping surface, in such a way that log I/log sin α is constant. For Series 1 each point is the mean of fifteen determinations; in Series 2, of twenty determinations.

uniformity. The two series of measurements are plotted together in Fig. 1, where it is obvious that they form a homogeneous whole.

Inasmuch as the variability of the response to the geotropic stimulus decreases in proportion to log sin α (Crozier and Pincus, 1926–27, *a*),

and since the photokinetic sideward movements of the head increase in proportion to log I (Crozier, 1926–27; Crozier and Pincus, 1926–27, *b*), it is to be expected that the two recognizable sources of variation in the apparent equilibrating light intensity must probably tend to counterbalance. The variability of the intensity required to equal the geotropic stimulation should therefore be fairly small. To test this, the probable errors of I have been computed (Table I); expressed as percentages of the mean intensities, which give figures corresponding to the coefficient of variation, it is seen that in agreement with

TABLE I.

Intensities of Light Required to Counterbalance Geotropic Excitation at Different Angles of Inclination (α) of the Creeping Plane.

	α	Log sin α	I, mean	Log I	P.E. of I, as per cent of mean
			foot candles		*per cent*
Series 1	15°	I.413	1.413	0.150	0.47
	20°	I.534	2.353	0.372	0.38
	30°	I.699	7.040	0.848	0.26
	40°	I.808	11.22	1.050	0.26
	50°	I.884	19.43	1.288	0.15
	60°	I.938	31.19	1.493	0.77
Series 2	15°	I.413	1.387	0.1421	0.35
	20°	I.534	2.968	0.4725	0.79
	35°	I.759	10.19	1.0082	0.44
	40°	I.808	11.45	1.0588	0.49
	50°	I.884	20.04	1.3019	0.41
	60°	I.938	28.08	1.4484	0.97
	70°	I.973	33.43	1.5241	0.83

this expectation the variability of I as measured is quite low. The quantity which inversely measures the photokinetic effect (Crozier, 1926–27) decreases linearly as log I increases, while the variability of geotropic orientation directly decreases as log sin α increases (Crozier and Pincus, 1926–27, *a*). Thus it is to be expected that the coefficient of variation for I in the present experiments should pass through a minimum as α increases from 10° to 70°. The differences among the probable errors in Table I are statistically significant. Fig. 2 shows that the variability of I, as α is increased, does pass through a minimum in the expected way.

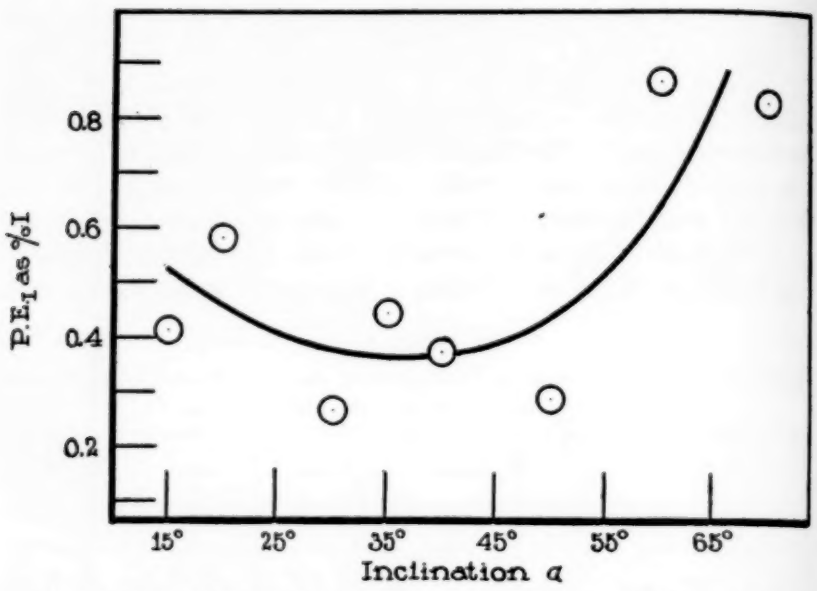

FIG. 2. The variability of the intensity required to counterbalance geotropic excitation passes through a minimum as the angle of inclination of the creeping surface is increased (see text).

IV.

SUMMARY.

The intensity of light required to just counterbalance geotropic orientation of young rats, with eyelids unopened, is so related to the angle of inclination (α) of the creeping plane that the ratio log I/log sin α is constant. This relationship, and the statistical variability of I as measured at each value of α, may be deduced from the known phototropic and the geotropic conduct as studied separately, and affords proof that in the compounding of the two kinds of excitation the rat is behaving as a machine.

CITATIONS.

Crozier, W. J., 1926–27, *J. Gen. Physiol.*, viii, 671.

Crozier, W. J., and Pincus, G., 1926, *Proc. Nat. Acad. Sc.*, xii, 612; 1926–27, *a*, *J. Gen. Physiol.*, x, 257; 1926–27, *b*, *J. Gen. Physiol.*, x, 407.

Pincus, G., 1926–27, *J. Gen. Physiol.*, x (in press).

CERTAIN EFFECTS OF SALTS ON THE PENETRATION OF BRILLIANT CRESYL BLUE INTO NITELLA.

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I.

INTRODUCTION.

The effect of salts on the rate of penetration of dye into living cells has been studied by various investigators[1] by comparing the rate in dye solution containing salt with that in dye solution containing no salt, but it is not possible to determine from their results whether the salt acts on the protoplasm or on the dye or on both.

The writer therefore has suggested a method[2,3] by which we are able to separate the effect of salt on the protoplasm from that on the dye. This is done as follows: (1) Cells are placed for a given time in a salt solution, after which they are placed in dye solution containing no salt. The rate of penetration of dye in the case of such cells is compared with that of the control (cells which have not been previously exposed to the salt solution). This gives the effect of salt on the protoplasm since it has previously been shown[2,3] that there is no error due to salt adhering to the surface of the cell wall (which cannot be removed by wiping and washing). It might be thought that an error could arise from the fact that salt might diffuse out from the cell when removed from the salt solution and placed in the dye solution

[1] *Cf.* Endler, J., *Biochem. Z.*, 1912, xlii, 440; xlv, 359. Szücs, J., *Sitzengsber. k. Akad. Wissensch., math.-naturw. Cl., Wien*, 1910, cxix, 1. These papers contain references to earlier literature. Lack of space prevents a detailed comparison of their results with those of the writer, but it may be said that in general they found that in some cases the presence of salts in the dye solution accelerated and in other cases inhibited the penetration of dye.

[2] Irwin, M., *Proc. Soc. Exp. Biol. and Med.*, 1926, xxiv, 54.

[3] Irwin, M., *J. Gen. Physiol.*, 1926–27, x, 271.

containing no salt, and enough of this might collect in the solution just outside the protoplasm to affect the results. This, however, has been shown[2,3] not to take place. (2) Cells previously exposed to water or salt solution are placed in dye solution containing the salt, and the rate of penetration in these cells is compared with that of the control (cells previously exposed to distilled water and then placed in dye solution containing no salt).

Preliminary accounts of such experiments have been given in the writer's previous papers.[2,3] These experiments are of interest because they are helpful in locating the factor controlling the rate of penetration of dye under various experimental conditions, which is one of the important problems in permeability.

The theory underlying the mechanism of the penetration of basic dye into living cells has been presented[4] by the writer and need not be discussed in detail here. But it may be stated that we assume that we are concerned only with the diffusion of the dye in the form of free base (for convenience called DB) into the vacuole, since the salt (called DS for convenience) does not enter the cell very readily. As DB enters[5] the vacuole, some of it changes to DS, and more DB enters until there is in the vacuole a definite ratio of DB/DS depending on the condition of the sap, *e.g.* the pH value and the salt content. At equilibrium, DB[6] in the vacuole is either equal to or proportional to DB in the external solution, depending on the solubility, etc.

II.

Methods.

Since the method of determining the amount of dye in the vacuole and the general experimental precautions and procedure have been repeatedly described in the writer's previous publications, a detailed account will not be given here.

The determination of the amount of dye in the sap was made colorimetrically. The dye, brilliant cresyl blue, was dissolved in M /150 borate buffer at pH 7.7 unless

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[4] Irwin, M., *J. Gen. Physiol.*, 1926–27, x, 75.

[5] The same may be said of DB in the protoplasm.

[6] What we actually measure is the total dye (DB and DS) but since the color of DB and DS is the same, and since there is a definite ratio of DB /DS, we are justified in using this method.

otherwise stated. The pH values of the solutions were determined by means of the hydrogen electrode. The experiments were carried out in an incubator at 25 ± 0.5°C.

Only living cells (collected in Cambridge unless otherwise stated) free from foreign growths, such as diatoms on the surface, were used and great care was taken to obtain uniform cells and to keep them all under the same conditions before experimentation. The cells were invariably wiped and washed in distilled water for 5 seconds, and again wiped, before they were placed in any solution.

Each experiment represents an average of about 60 readings on individual cells, and the probable error of the mean is less than 7 per cent of the mean.

In carrying out a series of comparative experiments, approximately the same number of readings was made for each experiment each day, and an average was taken of all the readings of each experiment after 60 readings were made. There may be a great source of error if comparative experiments are not made at the same time and with uniform cells.

III.

Salts with Monovalent Cations.

The experiments[7] (see Table I) with 0.013 M NaCl solution show that the rate of penetration of dye (as compared with that of control cells transferred from distilled water to the dye solution) decreases greatly when cells, after exposure to the salt solution for 10 minutes,

[7] The experiments were carried out as follows: Cells were divided into four groups:

(*a*) Cells were placed for 10 minutes in distilled water (pH 5.4), after which they were wiped and placed in 0.00014 M brilliant cresyl blue solution at pH 7.7 (M/150 borate buffer mixture). After 2 minutes the concentration of the dye in the sap was determined. This experiment represents the control, and this rate of penetration is used as a standard of comparison.

(*b*) Cells were exposed for 10 minutes in 0.013 M NaCl solution (made up in the distilled water), after which they were wiped and washed for 5 seconds in distilled water. Then they were again wiped and placed in the same dye solution as group *a*. After 2 minutes the concentration of dye in the sap was determined.

(*c*) Cells were placed in distilled water for 10 minutes, after which they were wiped, and placed in 0.00014 M dye solution containing 0.013 M NaCl at pH 7.7 (M/150 borate buffer mixture). After 2 minutes the concentration of dye in the sap was determined.

(*d*) Cells were exposed for 10 minutes in 0.013 M NaCl solution, after which they were wiped, and washed for 5 seconds in distilled water. Then they were again wiped and placed in the same dye solution as group *c*. After 2 minutes the concentration of dye in the sap was determined.

TABLE I.

Penetration of Brilliant Cresyl Blue into the Vacuole of Living Cells of Nitella in Presence of NaCl at Different Concentrations.

I. Concentration of external NaCl solution.

II. Concentration of dye in the vacuole when cells were placed in NaCl solution (Column I) for 10 minutes and then placed in 0.00014 M dye solution at pH 7.7 (borate).

III. Concentration of dye in the vacuole, when cells were placed in distilled water for 10 minutes and then in 0.00014 M dye solution at pH 7.7 (borate) containing the same concentration of salt as in I.

IV. Cells were placed for 10 minutes in the salt solution stated in I, and then placed in the same dye solution as in III.

As a basis of comparison the concentration of dye in the vacuole was taken when cells were placed in distilled water for 10 minutes and then placed in the same dye solution as in II. This concentration was 25.0×10^{-5} M.

In all cases the concentration of dye in the vacuole was determined after 2 minutes exposure to the dye solution.

I	Concentration of dye in the vacuole		
	II	III	IV
M	M × 10^5	M × 10^5	M × 10^5
0.0500	7.9	30.1	33.0
0.0250	7.2	29.3	32.2
0.0125	6.9	29.5	31.4
0.0063	7.6	29.3	30.5
0.0016	15.5	23.8	23.7
0.00063	22.8	24.5	24.0

TABLE II.

Concentration of brilliant cresyl blue in the vacuole of living cells of *Nitella* when cells previously exposed to tap water or for different lengths of time to distilled water or to 0.05 M or 0.01 M NaCl solution are placed in 0.00014 M dye solution at pH 7.7 (M/150 borate buffer mixture) for 2 minutes.

In tap water at pH 7.0	In distilled water pH 5.4		In NaCl solution				
	1/2 min.	10 min.	5 sec.	1/2 min.	2 min.	5 min.	10 min.
M × 10^5	M × 10^5	M × 10^5	M × 10^5	M × 10^5	M × 10^5	M × 10^5	M × 10^5
22.4	21.4	22.1	24.2	5.2	5.9	4.5	5.2

are subsequently placed in the 0.00014 M dye solution made up with borate buffer mixture at pH 7.7 for 2 minutes. Cells previously exposed either to distilled water or to 0.013 M NaCl for 10 minutes and then placed for 2 minutes in the same concentration of dye, at the same pH value, containing 0.013 M NaCl solution, show a slight increase in the rate of penetration of dye (Table I).

These experiments were repeated with different concentrations of salt between 0.05 M and 0.0006 M (Table I). Both the inhibiting and accelerating effects of NaCl remain about the same between 0.05 M

TABLE III.

Experiments Showing That the Effect of NaCl on the Protoplasm Causing a Decrease in the Rate of Penetration of Brilliant Cresyl Blue into the Vacuole of Nitella (Collected in New York) Is Not Readily Reversible.

I. Represents the control experiment. Cells placed for 10 minutes in distilled water and then placed in the dye solution.

II. Cells were exposed for 2 minutes to 0.0016 M NaCl solution after which they were placed in the dye solution.

III. Cells were exposed for 2 minutes to 0.0016 M NaCl after which they were washed in a large volume of distilled water for 1 hour, and then placed in the dye solution.

In every case the cells were placed for 1 minute in 0.00014 M dye solution at pH 7.7 (M/150 borate buffer mixture).

	I	II	III
	M × 10^5	M × 10^5	M × 10^5
Amount of dye in sap.....	31.8	18.9	20.0

and 0.006 M, but below the latter concentration both effects diminish as the concentration decreases until at 0.0006 M solution there is practically no more effect.

This inhibiting effect of NaCl may be brought about in ½ minute at 0.01 M or above (Table II). This effect is not removed by transferring the cells after 2 minutes from the salt solution to distilled water and leaving them for 1 hour, as shown in Table III.

When the experiments were repeated with KCl, KNO_3, LiCl, Na citrate, and Na_2SO_4 at 0.01 M solution the same results were obtained as is shown in Table IV.

TABLE IV.

Penetration of Brilliant Cresyl Blue into the Vacuole of Living Cells of Nitella in the Presence of Salts.

I. Cells were exposed for 10 minutes to 0.01 M salt solution after which they were placed in the 0.00014 M dye solution at pH 7.7 (M/150 borate buffer mixture).

II. Cells were exposed for 10 minutes to distilled water after which they were placed in the 0.00014 M dye solution at pH 7.7 (M/150 borate buffer mixture) containing 0.01 M salt.

III. Cells were exposed for 10 minutes to 0.01 M salt solution after which they were placed in the same dye solution as in II.

As a basis of comparison the concentration of dye in the vacuole was taken when the cells were placed in distilled water for 10 minutes and then transferred to the same dye solution as in I. This concentration was 24.2×10^{-5}M.

In all cases the cells were placed in the dye solution for 2 minutes.

0.01 M external salt solution.	Concentration of dye in the vacuole		
	I	II	III
NaCl	6.9	28.1	31.0
LiCl	7.9	27.8	32.5
KCl	8.3	29.0	31.3
KNO_3	7.3	27.5	30.0
Na_2SO_4	5.8	29.5	32.2
Na citrate	5.5		
$CaCl_2$	26.0	26.2	26.5
$MgCl_2$	26.2	25.9	27.0
$MgSO_4$	25.8	26.5	26.8
$LaCl_3$	29.2		
$La(NO_3)_3$	28.3		

IV.

Salts with Bivalent and Trivalent Cations.

When the experiments[8] were carried out using salts with bivalent and trivalent cations, namely,[9] $MgCl_2$, $CaCl_2$, $MgSO_4$, $LaCl_3$, and

[8] These experiments were carried out by dividing the cells into four groups in the same manner as with NaCl, as described in Foot-note 7, which should be consulted for the details of the experiments.

[9] $LaCl_3$ and $La(NO_3)_3$ reduced the pH value of the solution considerably so that the experiments were carried out with lower concentrations of the salt and the same result was obtained.

$La(NO_3)_3$, it was found that there is a very slight accelerating effect in the case of cells previously exposed to the salt solution and then placed in the dye solution containing either salt or no salt, as well as in the case of cells directly transferred from distilled water to the dye solution containing salt (Table IV).

TABLE V.

Experiments Showing That the Effect of NaCl on the Protoplasm Causing a Decrease in the Rate of Penetration of Brilliant Cresyl Blue into the Vacuole of Living Cells of (New York) Nitella May Be Removed by Salts with Bivalent and Trivalent Cations.

The cells were left for 5 minutes in each salt solution, and then placed in 0.00014 M dye solution at pH 7.7 (M/150 borate buffer mixture) for 1 minute.

The cells were exposed to the various solutions in the order given. The cells kept in distilled water for 5 minutes and then transferred to the dye solution for 1 minute had 28×10^{-5} M dye in the vacuole.

The cells exposed to 0.001 M NaCl for 5 minutes and then transferred to the dye solution had 15.6×10^{-5} M dye in the vacuole.

Salt solution	0.001 M NaCl, then salt solution, then dye	Salt solution, then 0.001 M NaCl, then dye
	M × 10^5	M × 10^5
0.01 M $MgCl_2$	29.5	16.1
0.01 M $MgSO_4$	30.0	16.6
0.001 M $MgSO_4$	29.2	15.0
0.001 M $LaCl_3$	28.4	17.0

In considering the results obtained with all the salts given above, we may conclude that the decreasing effect[10] of the salts with monovalent cations is due to the effect of the cations and not to that of the anions, while the bivalent and trivalent cations have no inhibiting effect on the protoplasm.

[10] For results with certain concentrations of dye and salts (including a decrease in penetration of dye in dye solution containing either 0.05 M $CaCl_2$ or $MgCl_2$ at higher pH value). See Irwin, M., *Proc. Soc. Exp. Biol. and Med.*, 1926-27, xxiv, No. 4.

V.

The Inhibiting Effect of NaCl May Be Removed by Salts with Bivalent and Trivalent Cations.

The question now arises whether the inhibiting effect of NaCl may be entirely or partially removed by other salts. To test this question the following experiments were made.

Cells were exposed to 0.001 M NaCl solution for 5 minutes after which they were washed in 0.01 M $MgCl_2$ for 5 minutes, and then transferred to the dye solution. The inhibiting effect of NaCl was found to have been completely removed[11] since the rate of penetration of dye was the same as that of the control, as shown in Table V. This inhibiting effect, however, was not removed when cells were first exposed to the $MgCl_2$ solution and subsequently treated with NaCl and then placed in the dye solution (Table V)

The same result is obtained (Table V) when $LaCl_3$ and $MgSO_4$ are used instead of $MgCl_2$.

No inhibiting effect of NaCl is produced if cells are exposed to 0.02 M NaCl containing 0.01 M $MgCl_2$ and then placed in the dye solution. In some cases the inhibiting effect of NaCl is removed in dye made up with phosphate buffer mixture, or with borate buffer mixture at high pH value in which borax predominates, or at lower pH value containing NaCl or $MgCl_2$.

Theoretical Considerations.

The experimental results described above appear to involve two factors, (1) effect of salt on the dye, (2) action of salt on the protoplasm (either at the surface or in the interior).

A salt (with monovalent, bivalent, or trivalent cations) may affect the dye in three ways, (*a*) increase the dissociation of the dye, (*b*) decrease the solubility of the dye, (*c*) have some other (possibly specific) effect on the dye.

In order to study the effect of salt on the dye experiments were made on the distribution of the dye between chloroform and the 0.00014 M

[11] The inhibiting effect brought about by 0.01 M NaCl may also be removed by phosphate buffer mixture containing 0.00014 M dye, while it cannot be removed by the buffer solution containing no dye.

dye solution (borate buffer mixture) at pH 7.7. These show that the addition of 0.01 M NaCl or 0.01 M $MgCl_2$ to the dye solution does not increase the amount of dye taken up by the chloroform as would be the case if the dye were being salted out but on the contrary decreases it, which may indicate that the solubility of the dye (at such concentrations of the dye and the salts as are here employed) is not affected as much as the dissociation constant of the dye. The salt appears to increase the dissociation of the dye which would cause less dye to enter the chloroform.

The question arises whether we can apply these results directly to *Nitella*. In view of the fact that the rate of penetration of the dye at various pH values was previously found[4] by the writer to correspond with the amount of dye absorbed by the chloroform from aqueous solution at these pH values, we might expect a similar correspondence to exist when cells are placed in the dye solution containing the salt, but the experiments show the contrary. Less dye is taken up by the chloroform while more dye is taken up by the cells when the aqueous dye solution contains salt. This may indicate that the effect of change in solubility exceeds that of the shift in dissociation constant, in the case of *Nitella*, since the partition coefficient of DB between the cell vacuole and the external solution is small, probably less than 1. Hence a slight change in the solubility of DB in the external solution may bring about a great change in the partition coefficient, while the partition of DB between chloroform and M/150 buffer solution is found to be exceedingly large so that a slight change in the solubility may have no appreciable effect on the partition coefficient. But it does not seem possibe to explain these results on *Nitella* entirely on the basis of solubility because the salting out effect of a salt with monovalent cation, such as NaCl, would probably be less than that of a salt with bivalent cation, such as $MgCl_2$, so that at the same concentrations one might expect less accelerating effect with NaCl than with $MgCl_2$, but the experiments show that NaCl brings about a greater effect than $MgCl_2$ (which has very little effect) which indicates that the situation may be complicated by additional factors such as the specific effect of cations on the dye and on the protoplasm.

That the accelerating effect in dye plus NaCl is due to the direct

action of the salt on the dye in the external solution and not on the protoplasm is shown by the experiment where the cells are exposed to the salt solution and then placed in the dye solution containing no salt, in which case there is a decrease in the rate of penetration of dye instead of an increase. This decrease cannot be due to the entrance of salt into the vacuole since the halide content remains unchanged. It must therefore be due to the presence of the salt in the protoplasm (either at the surface or in the interior). We may then assume that the salt acts on the protoplasm in such a way that the rate of penetration of dye from the external solution into the protoplasm is decreased (it might, for example, decrease the solubility of dye in the protoplasm or increase the viscosity of the protoplasm) and if the penetration of dye into the outer surface of the protoplasm is slower than the diffusion of the dye from the protoplasm into the vacuole we may expect a decrease in the amount of dye in the vacuole.

A striking fact is that this inhibiting effect not only disappears but is replaced by an accelerating effect when cells thus exposed to the salt solution are placed in the dye solution containing salt. The accelerating effect is a little greater in this case than when cells are first exposed to distilled water and then placed in the same dye solution. It has been shown by experiments that at the concentration of NaCl employed the effect of the salt on the protoplasm is immediate so that irrespective of whether the cells have been formerly exposed to the salt or not the condition of the protoplasm may be practically the same very soon after the cells are placed in the dye solution containing the salt.

If this is true we may assume that the reason the accelerating effect is a little greater in the case of the cells previously exposed to the salt is because there is a greater accumulation of salt in the very thin film of liquid between the protoplasm and the cell wall and that this acts on the dye.

It would seem that this accelerating effect is due to a change in the nature of the dye (produced by the salt) which makes the dye penetrate more rapidly. It may not be necessary to suppose that the combination of salt plus dye affects the protoplasm so as to increase

penetration because cells exposed[12] to such a combination behave subsequently like those exposed to salt without dye (showing a decrease in the rate of penetration when placed in the dye). We may therefore suggest that the effect of salt on the protoplasm is not affected by presence of dye. Further experiments are necessary, however, to determine this conclusively.

In striking contrast to the behavior of NaCl is that of salts with bivalent and trivalent cations. When cells are exposed to a solution of $MgCl_2$ and then placed in the dye solution there is no inhibiting effect such as is produced by previous exposure to NaCl. On the contrary, there is apparentiy an accelerating effect but this is so small that it may possibly be due to experimental error.

There is another difference[13] between these salts. The inhibiting effect of NaCl may be reversed by adding $MgCl_2$ to the solution containing NaCl or by washing the cells with a solution of $MgCl_2$ after they have been exposed to the solution of NaCl.

SUMMARY.

The effect of various substances on living cells may be advantageously studied by exposing them to such substances and observing their subsequent behavior in solutions of a basic dye, brilliant cresyl blue.

The rate of penetration of the basic dye, brilliant cresyl blue, is

[12] From the time curves it is evident that the same amount of accelerating effect is produced whether the cells are placed in 0.02 M NaCl solution at pH 7.7 (borate buffer mixture) containing 14×10^{-5} M or 1.7×10^{-6} M dye. An inhibiting effect is produced when cells are exposed for 3 minutes to 0.02 M NaCl solution at pH 7.7 (borate buffer mixture) containing either 1.7×10^{-6} M dye or no dye, and then placed in 14×10^{-5} M dye solution at the same pH value for 1 minute.

[13] These differences may result from the fact that each salt affects the protoplasm or the dye in more than one way so that the net result may differ because with one salt one effect is of primary importance while with another salt a different effect predominates. One salt may affect diffusion from the external solution into the protoplasm a great deal while another may have more effect on the diffusion from the protoplasm into the vacuole and the location of these effects may change with alterations in concentration. The same may be said of differences in viscosity, solubility, etc.

decreased when cells are exposed to salts with monovalent cations before they are placed in the dye solution (made up with borate buffer mixture). This inhibiting effect is assumed to be due to the effect of the salts on the protoplasm.

This effect is not readily reversible when cells are transferred to distilled water, but it is removed by salts with bivalent or trivalent cations. In some cases it disappears in dye made up with phosphate buffer mixture, or with borate buffer mixture at the pH value in which the borax predominates, and in the case of NaCl it disappears in dye containing NaCl.

No inhibiting effect is seen when cells are exposed to NaCl solution containing $MgCl_2$ before they are placed in the dye solution.

The rate of penetration of dye is not decreased when cells are previously exposed to salts with bivalent and trivalent cations.

The rate is slightly increased when cells are placed in the dye solution containing a salt with monovalent cation and probably with bivalent or trivalent cations. In the case of the bivalent and trivalent salts the increase is so slight that it may be negligible.

THE STAGES OF THE PEPTIC HYDROLYSIS OF EGG ALBUMIN.

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Numerous investigators have studied the stages in peptic digestion. The conclusions at which they have arrived may be grouped under two main heads.

On the one hand, Kühne (1) and Neumeister (2) concluded that the products of digestion arise by a serial degradation from the protein through acid albumin to proteose to peptone. On the other hand, Lawrow (3), Klug (4), Pick (5), Zunz (6), and Goldschmidt (7) found that the products of digestion appear more or less simultaneously.

The most comprehensive study of the stages of peptic digestion was made by Zunz. His general conclusion was that metaprotein, proteoses (primary and secondary), peptones, and simpler products, arise simultaneously and by inference directly from the protein molecule. The metaprotein and a large part of the primary proteose, he found to be converted into peptones. He also described large amounts of substances (61 per cent of the total nitrogen) simpler than peptones which appeared in the early hours of digestion and later decreased.

Klug concluded that the acid metaprotein formation in the early stages of digestion was due to the action of pepsin, while Maly and Boos (8) believed that it might be due to the action of acid alone.

A reexamination of the problem seemed desirable. The methods used were chiefly those described by Wasteneys and Borsook (9) for the fractional analysis of incomplete protein hydrolysates.

Metaprotein (acid albumin) was estimated by determination of the nitrogen content of the precipitate obtained on careful adjustment of the reaction to pH 6.0.

The proteose was precipitated by saturation of the solution with

sodium sulfate, at 33°C., and the peptone by precipitation with tannic acid under specially controlled conditions. The tannic acid filtrate was precipitated with alcohol and zinc sulfate. The nitrogen content of this precipitate was taken as subpeptone nitrogen, and the remainder as residual nitrogen, contained, it is assumed, in the simplest constituents of the hydrolysate.

No attempt was made to subdivide proteoses into primary and secondary classes except in one phase of the investigation which will be discussed later.

The results obtained are in accordance with those of Zunz and Pick that most of the products found in a peptic hydrolysate arise directly from the protein molecule. They differ in regard to the significance of acid metaprotein, and also in regard to the amount of, and variations in, the fraction simpler than peptone.

It was found that acid metaprotein is hydrolyzed more slowly than the native albumin from which it is derived; that the peptic hydrolysis of albumin can be effected at acidities less than pH 4.0, where no formation of metaprotein can be demonstrated; and that the hydrogen ion concentration for the minimum hydrolysis of undenatured albumin is distinctly different from that of acid metaprotein. For the former it is in the neighborhood of pH 6.0; for the latter at pH 4.0. From these results it was concluded that acid metaprotein formation, while a product of the action on protein of acid alone, as is well known, is not a necessary stage in the peptic hydrolysis of albumin, and its influence, if any, on hydrolysis, is to retard it.

Two-thirds of the initial amount of protein is hydrolyzed in 4 hours. The reaction then becomes progressively slower, so that in 12 hours 10 per cent, and in 7 days 6 per cent of the protein remains still unhydrolyzed. This rate of hydrolysis, however, holds only under the conditions of this experiment, but the rapid initial decomposition is characteristic of peptic hydrolysis under any conditions.

The results indicate that the peptic hydrolysis of albumin progresses in two stages. The first stage, occurring in the first 12 hours, consists of the hydrolysis of practically 100 per cent of the protein, with the formation of products of which 85 per cent may be spoken of as primary, *i.e.* undergo no further hydrolysis. The products at

this time consist of 55 per cent proteose, 17 per cent peptone, 12 per cent subpeptone, and 5 per cent residual nitrogen. A second stage, occurring later and progressing much more slowly, results in the hydrolysis of 15 per cent of the primary products into simpler fragments, which may be designated as secondary. The secondary hydrolysis occurs in both the proteose and subproteose fractions.

No methods are available for following subsequent changes in the subproteose fraction. In any case they affect the main picture of peptic hydrolysis very little, as only a very small fraction, about 6 per cent at most, of the total N is involved.

EXPERIMENTAL.

Metaprotein Formation and Its Relation to Hydrolysis.

Egg albumin (Merck) and pepsin (Merck) have been employed throughout. The metaprotein was prepared by allowing a solution of albumin at pH 1.6 to stand at room temperature for several days, thymol being employed as antiseptic.

The rate of formation of metaprotein is shown in Table I. After several weeks it attains a value of about 80 per cent of the total N, leaving approximately 15 per cent of the nitrogen still in the form of albumin soluble at neutrality.

A solution of metaprotein, on the other hand, prepared by solution at pH 1.6 of the precipitate obtained at pH 6.0 remains unchanged after standing for days at room temperature.

To compare the relative rates of hydrolysis by pepsin of metaprotein, and of albumin which has not been denatured by acid, two simultaneous hydrolyses were followed. The first hydrolysis was of an albumin solution which had been standing at room temperature at pH 1.6 for several days. The second was of an albumin solution of equal nitrogen content, to which the enzyme was added simultaneously with an amount of acid necessary to bring the pH to 1.6. This experiment was performed with 2.0, 1.5, 1.0, 0.5, and 0.25 per cent stock pepsin solutions, lasting 45 minutes. Considering the lowest enzyme concentration as 1 unit the others were, respectively, 10, 8, 6, 4, and 2 units. The results are recorded in Fig. 1.

It is clear from Fig. 1 that in mixtures of undenatured albumin and acid metaprotein, the velocity of peptic hydrolysis is greater where the concentration of acid metaprotein is less. Fig. 1 also shows the surprising result, that, at least in these experiments, strict proportionality between velocity of hydrolysis and concentration of pepsin obtains in those solutions where the conversion of undenatured protein to metaprotein has attained approximate equilibrium.

The slower rate of hydrolysis of acid metaprotein, whether the solution consists almost entirely of metaprotein, as in the previous experiment, or of a mixture of

metaprotein and undenatured albumin, is shown in an experiment the result of which is indicated in Fig. 2. In this experiment the relative rates of hydrolysis were compared in three solutions of identical nitrogen and enzyme content, one containing undenatured albumin, a second consisting of equal parts of undenatured albumin and acid metaprotein, and a third containing only metaprotein. As the curves show, peptic hydrolysis is most rapid in the undenatured albumin solution, and least rapid in the metaprotein solution, while the relative rate of hydrolysis of the mixture is intermediate.

Since acid metaprotein was hydrolyzed more slowly than undenatured albumin, it seemed probable that the formation of metaprotein indeed was not, as Maly, and Boos (8) maintained, a necessary stage in peptic hydrolysis. To test this, experiments were performed to ascertain the possibility of obtaining peptic hydrolysis of albumin at hydrogen ion concentrations where no metaprotein is formed.

TABLE I.

Rate of Formation of Acid Metaprotein from Egg Albumin at pH 1.6 and at 20°C.

Time	Metaprotein
	per cent total N
1 min.	10.6
5 "	17.0
20 "	18.9
30 "	21.3
1 hr.	26.5
2 "	33.3
6 "	39.0
1 day	54.6
3 "	56.9
5 "	68.6

It was necessary first to define the limit of acidity beyond which metaprotein formation does not occur. Portions of a neutral solution of 3.2 per cent albumin were adjusted to various acidities with hydrochloric acid. These were set away in a water bath at 37.7°C. for 45 minutes. They were then assayed for metaprotein by precipitation at pH 6.0. The results are given in Table II. They show that no metaprotein formation occurs at acidities less than pH 4.0.

As a result of this experiment a number of hydrolyses were carried out at acidities slightly less than pH 4.0 with several concentrations of pepsin. Typical results are given in Table III.

These experiments show that it is possible to effect considerable amounts of hydrolysis without the formation of any metaprotein. They also show that when hydrolysis is measured by following the

changes occurring in the protein substrate, it is possible to detect the progress of hydrolysis at acidities less than pH 4.0. Michaelis (10) found that peptic hydrolysis of albumin ceases at pH 4.0.

The reason for the difference between our findings and those of Michaelis lies in the probability that the latter worker was dealing

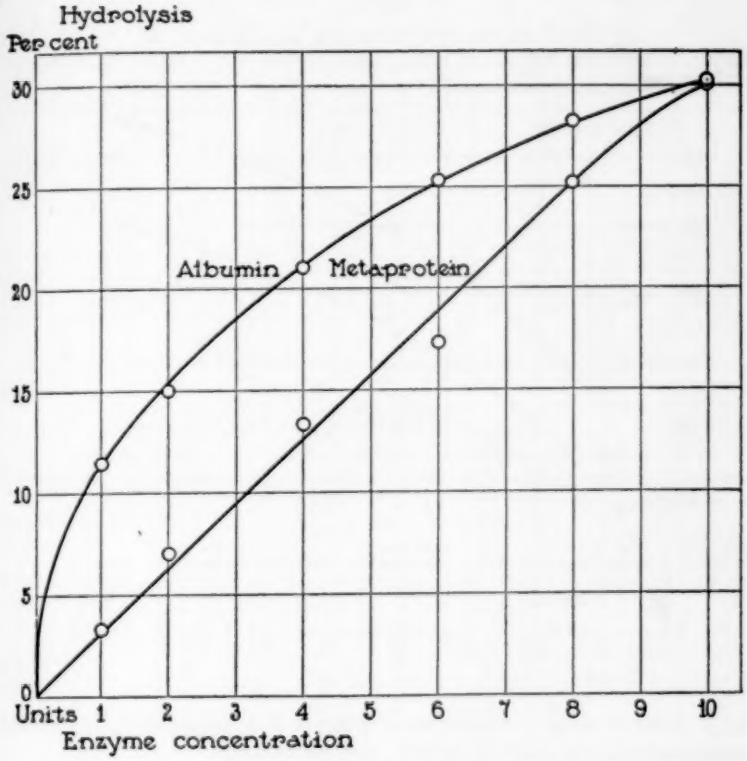

FIG. 1. Relative rates of hydrolysis of albumin and metaprotein.

with a solution consisting largely of metaprotein. The hydrogen ion concentration for the threshold of minimum hydrolysis of acid metaprotein is at pH 4.0; for egg albumin, which has not been denatured, it is in the neighborhood of pH 6.0. The optimum pH is approximately the same for both, near pH 1.6. This is shown in Fig. 3. In this experiment 3.2 per cent solutions of albumin and of acid

metaprotein were hydrolyzed for 1 hour at 37.7°C. with 0.2 per cent pepsin at the acidities indicated.

In the peptic hydrolysis of coagulated egg albumin observations were made, which, in contradistinction to the findings with uncoagulated albumin, do not conform to the view that acid metaprotein is not a necessary stage in the peptic hydrolysis of albumin. A 3.2

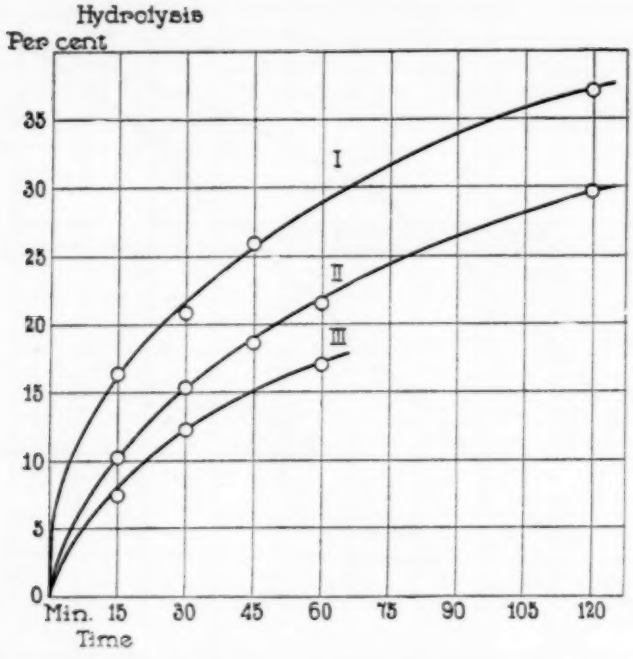

FIG. 2. Relative rates of hydrolysis of albumin and metaprotein. I, albumin; II, mixture of albumin and metaprotein; III, metaprotein.

per cent solution of albumin, acidified with dilute acetic acid, was coagulated by boiling. The coagulate was filtered and washed thoroughly with distilled water. It was then suspended in dilute HCl, and the reaction of the suspension adjusted to pH 1.6. Pepsin was added to a concentration of 0.2 per cent, and the suspension was incubated at 37.7°C. for 3 hours. At the end of this time all of the coagulum had disappeared. Part of the digest was neutralized;

another part was treated with trichloroacetic acid. In the neutralized solution a heavy precipitate appeared. The total nitrogen of the solution was 42 mg. The nitrogen in the filtrate from trichloroacetic acid precipitation was 30.6 mg. indicating that 11.4 mg. of protein nitrogen had not been hydrolyzed. The filtrate of the neu-

TABLE II.

Relation of Acid Metaprotein Formation to the C_{H^+}.

pH	Amount of metaprotein formed
	per cent total N
6.0	0
4.5	0
4.3	0
4.2	0
4.1	0
4.0	0
3.9	3.9
3.5	21.6

TABLE III.

Peptic Hydrolysis of Egg Albumin at Acidities Less than pH 4.0.

Enzyme concentration	pH 4.1		pH 4.2	
	Amount of hydrolysis	Metaprotein formed	Amount of hydrolysis	Metaprotein formed
units	*per cent*		*per cent*	
1	5.3	0	2.8	0
2	6.3	0	—	—
4	6.5	0	—	—
6	8.2	0	3.4	0
8	10.6	0	—	—
10	10.7	0	4.4	0

tralized solution contained 31.9 mg. of nitrogen. The precipitate thrown out on neutralization contained, therefore, 10.1 mg. The nitrogen precipitated by trichloroacetic acid was 11.4 mg., by neutralization 10.1 mg. In view of the difference of the two methods the correspondence is sufficiently close to indicate that the same amount of material was precipitated by neutralization and by tri-

chloroacetic acid. Evidently there is a material produced in the early stages of the hydrolysis of coagulated egg albumin which is soluble in dilute acid, insoluble at the neutral point, and precipitated by trichloroacetic acid. This agrees with the definition of metaprotein. We are led to the conclusion, for the time being, that though metaprotein is not a stage in the peptic hydrolysis of soluble unde-

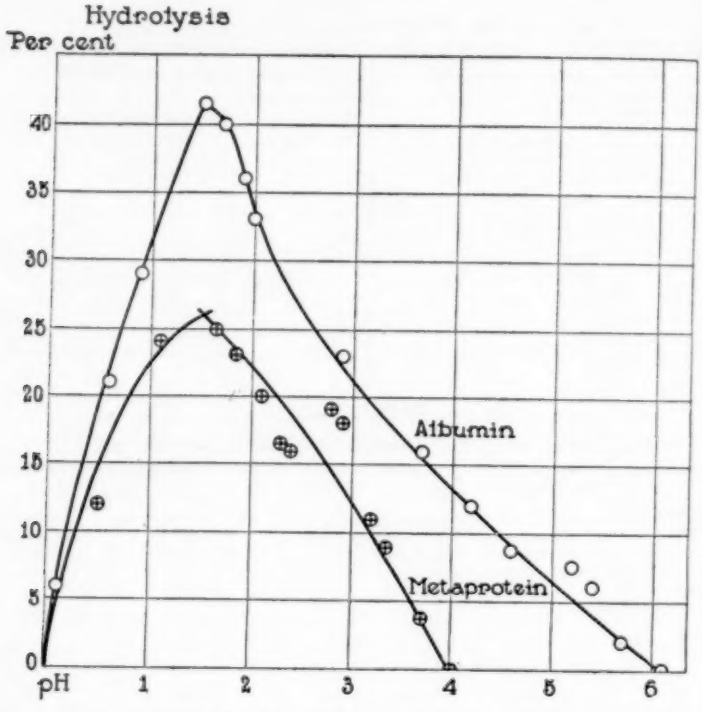

FIG. 3. Relative effect of C_{H^+} on the rates of hydrolysis of albumin and metaprotein.

natured albumin, it is probably the first stage in the hydrolysis of coagulated egg albumin.

The phenomena associated with heat coagulation of albumin have been fully discussed by Wu and Wu (11). This experiment, however, discloses an interesting anomaly. It is ordinarily supposed that in the coagulation of egg white by heat, albumin passes through the

stage of acid metaprotein. On suspending the coagulum in acid so that the reaction of the suspension is pH 1.6, contrary to the behaviour of uncoagulated albumin, no metaprotein is formed. Immediately on adding pepsin, however, metaprotein appears. Either the change from metaprotein to coagulated egg white is a reversible phenomenon with pepsin as a necessary adjuvant for reversion, or else we are dealing with different kinds of metaprotein.

The Stages in the Peptic Hydrolysis of Albumin.

The method of analysis was that described by Wasteneys and Borsook (9) for the fractional analysis of incomplete protein hydrolysates. Two types of experiments were carried out. One was a preliminary survey, in which the pH of the digest was allowed to rise with the progress of the hydrolysis. In the other, the pH was kept approximately constant at 1.6 throughout the course of the experiment by the occasional addition of acid. The findings in both types of hydrolyses were essentially the same, but the latter experiment was more thoroughly controlled, and the results there obtained are taken as the basis for the present discussion.

6 litres of a 5 per cent solution of egg albumin (Merck) were adjusted to pH 1.6 and sufficient solution of scale pepsin (Merck), also adjusted to pH 1.6, was added to make the concentration of enzyme 0.2 per cent.

The following control experiments were carried out. 1000 cc. of the albumin solution at pH 1.6 was precipitated with trichloroacetic acid and analyzed at the same time as the first sample from the digest. A simultaneous analysis was made of another 1000 cc. of the same albumin solution at pH 1.6 which had been in the incubator with the main digest for a week. These two controls determined the effect of the acid alone (pH 1.6) on egg albumin during the period of the experiment (168 hours). The protein N diminished from 96 per cent of the total nitrogen to 94 per cent. The proteose and residual nitrogen fractions remained unchanged. The peptone rose from 2 to 3 per cent and the subpeptone from 0 to 0.5 per cent. The proteolytic effect of the acid is therefore negligible. This is in accord with the conclusions of Frankel (12) who found no significant increase

in the free amino nitrogen of egg white after incubation for 96 hours with 0.2 per cent HCl at 38–40°C.

In the presence of pepsin, as Table IV shows, the hydrolysis of the protein was very rapid. In 4 hours more than two-thirds of the protein was hydrolyzed; in 12 hours 11 per cent of the original total amount of substrate remained. After this point the hydrolysis of the remaining protein proceeded slowly. For the complete hydrolysis of 3 per cent albumin at 37°C. with this enzyme concentration, between 2 and 3 weeks are required.

The proteose fraction attained a maximum value of 55 per cent. Between 12 and 28 hours, the amount of this fraction falls from 55

TABLE IV.

Fractional Analyses of a Peptic Digest of Albumin during the Course of Almost Complete Hydrolysis.

Duration of hydrolysis	Protein N	Proteose N	Peptone N	Subpeptone N	Residual N
	Per cent of original protein N				
3 min.	85	6	6	2	1
12 hrs.	11	55	17	12	5
28 "	5	50	29	15	2
48 "	5	46	29	18	5
72 "	4	45	30	19	5
96 "	5	45	31	17	5
168 "	6	40	27	23	7

per cent to 50 per cent; and in addition there is a further hydrolysis of 6 per cent protein. During this interval the peptone fraction gains 12 per cent, from 17 per cent to 29 per cent, while the subpeptone value increases only 3 per cent. The increase in residual nitrogen is negligible. The relatively greater gain in the peptone fraction is better shown by the curves, Fig. 4. At 12 hours, when the proteose curve has attained its peak, the slope of the subpeptone curve changes from a relatively steep ascent to a very slowly rising straight line. The peptone curve, on the other hand, continues to rise fairly quickly until 28 hours, when it flattens out. After 28 hours the proteose decreases from 50 per cent to 40 per cent; the peptone curve is practically flat, while the subpeptone rises from 15 to 23 per cent.

The peptone fraction rises rapidly to a value of 24 per cent in 28 hours. The increase between 12 and 28 hours is probably due, as discussed above, to the hydrolysis of proteose during this interval. After 28 hours the value of the peptone remains practically constant at approximately 24 per cent. This value probably represents an

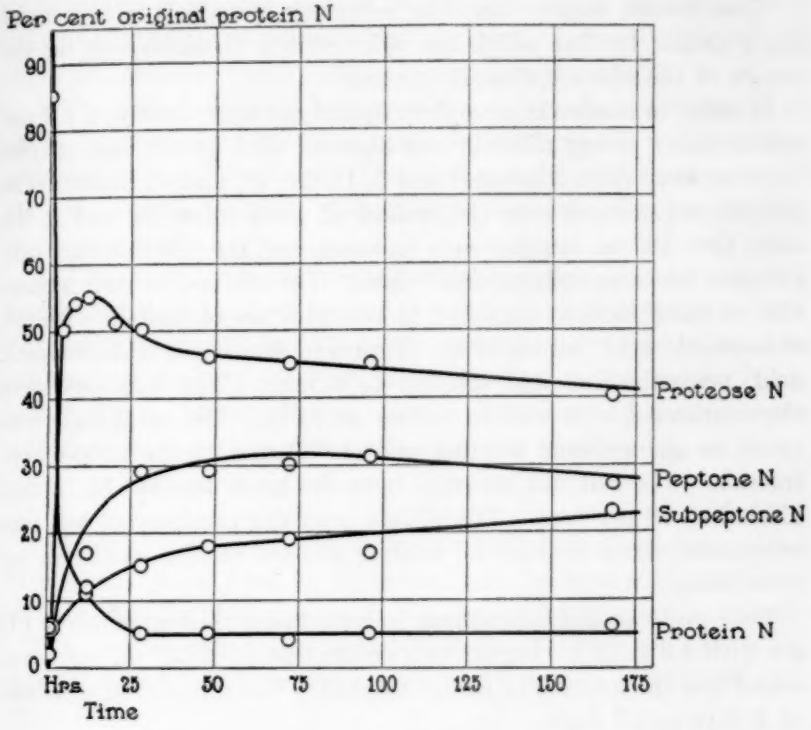

FIG. 4. Fractional composition of the hydrolysate during the course of almost complete peptic hydrolysis of egg albumin.

equilibrium between the amount gained from the hydrolysis of proteose and that lost by secondary hydrolysis of the peptone itself.

The subpeptone rises to a value of 10 per cent in 12 hours. The subsequent increase is slow and becomes significant only in the later periods of the hydrolysis, rising to 19 per cent in 168 hours. There is probably very little, if any, subpeptone hydrolyzed by pepsin. Hence

this fraction continues to increase steadily, gaining, by secondary hydrolysis, from the proteose and peptone.

The increase in residual nitrogen, though small, from 1 to 4 per cent, was obtained in a number of digests, and is larger than can be accounted for as experimental error. As Table IV shows, an increase in residual nitrogen is obtained very early in the hydrolysis.

These results suggest that the secondary hydrolysis is of a serial type, similar to that which the older writers considered to be the nature of the whole hydrolytic process.

In order to study the secondary hydrolysis more closely, a 3.2 per cent solution of egg albumin was digested with pepsin, and samples were removed after 3 minutes and 6, 12, 24, 48, and 72 hours. The protein and proteose were determined at these intervals, and at the same time 500 cc. samples were removed, and the proteose and subproteose fractions isolated from them. The method for the separation of these factions consisted in precipitation of protein with trichloroacetic acid, boiling of the filtrates to decompose trichloroacetic acid, neutralization, and dilution to volume. The solutions were then saturated with sodium sulfate at 33°C. The precipitate was taken as the proteose fraction and the filtrate as the subproteose fraction. The salt was removed from the latter fraction by adding alcohol to 50 per cent. The filtrate from the precipitated salt was evaporated down to drive off alcohol, and the residue was made up to volume.

Both proteose and subproteose fractions were then adjusted to pH 1.6 with 2.0 N HCl. Pepsin to a concentration of 0.2 per cent was added and the increase in formol titrable N was followed at intervals of 3 days and 7 days.

It is generally agreed in the literature that copper acetate is a specific precipitant for primary proteose. Both proteose and subproteose fractions were therefore treated with copper acetate according to the method of Folin (13), at intervals of 3 days and 7 days, simultaneously with the formol titrations.

Considering first the fraction precipitated by copper acetate, it was found to be present in both proteose and subproteose, which makes the specific nature of the copper acetate precipitation doubtful. However, the amount of N so precipitated in the proteose fraction

removed from the earlier stages of the primary hydrolyses was greater than in the fractions removed later, and to an extent that might account for the total falling off in proteose from the maximum attained during the primary hydrolysis. It is significant in this regard that there is no change in the N precipitated by copper acetate in any of the subproteose fractions.

From this it may be inferred that though copper acetate is not a specific precipitant of primary proteose, yet it appears to precipitate all of the proteose which undergoes secondary hydrolysis.

In the second digestion by pepsin the changes in free amino N, as shown by the formol titration, were relatively greatest in the earliest obtained proteose fractions. The later samples of proteose and all the subproteose fractions gave relative increases of approximately equal magnitude.

The conclusions are that secondary hydrolysis takes place in both fractions. It occurs to a greater extent in the earlier proteose fractions coinciding with the fall in the proteose curve. In relation to the main hydrolysis, however, these increases in free amino N, in both fractions, are small, and indicate the minor importance of the secondary hydrolysis in the peptic digestion of albumin.

SUMMARY.

1. Most of the products of the peptic hydrolysis of albumin, about 85 per cent of the total N, are primary in the sense that they arise directly from the protein molecule, and undergo no further hydrolysis.

2. A slow secondary hydrolysis, involving about 15 per cent of the total N, occurs in the proteose and simpler fractions primarily split off.

3. Acid metaprotein in peptic hydrolysis arises as a result of the action of acid. It is not an essential stage in the hydrolysis of undenatured albumin.

4. Acid metaprotein is hydrolyzed by pepsin more slowly under comparable conditions than undenatured albumin.

BIBLIOGRAPHY.

1. Kühne, W., and Chittenden, R. H., *Z. Biol.*, 1883, xix, 159; 1884, xx, 11; 1886, xxii, 423. Wenz, J., *Z. Biol.*, 1886, xxii, 1.

2. Neumeister, R., *Z. Biol.*, 1887, xxiii, 381; 1888, xxiv, 267; 1890, xxvi, 57, 324.

3. Lawrow, D., *Z. physiol. Chem.*, 1898–99, xxvi, 513; 1901, xxxiii, 312.
4. Klug, F., *Arch. ges. Physiol.*, 1895, lx, 43; 1897, lxv, 330.
5. Pick, E. P., *Z. physiol. Chem.*, 1898, xxiv, 246; 1899, xxviii, 219.
6. Zunz, E., *Z. physiol. Chem.*, 1899, xxvii, 219.
7. Goldschmidt, F., Inaugural dissertation, Strassburg, 1898, quoted from Hammarsten, O., Lehrbuch der physiolgischen Chemie, Munich, 10th edition, 1923, 101.
8. Maly and Boos, in Klug, F., *Arch. ges. Physiol.*, 1895, lx, 66.
9. Wasteneys, H., and Borsook, H., *J. Biol. Chem.*, 1924–25, lxii, 1.
10. Michaelis, L., Die Wasserstoffionenkonzentration, Berlin, 1914, 71.
11. Wu, H., and Wu, D. Y., *J. Biol. Chem.*, 1925, lxiv, 369.
12. Frankel, E. M., *J. Biol. Chem.*, 1916, xxvi, 31.
13. Folin, O., *Z. physiol. Chem.*, 1898, xxv, 152.

THE AUTODESTRUCTION OF PEPSIN IN RELATION TO ITS IONIZATION.

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The irreversible disappearance of the activity of pepsin in alkaline solution was first recorded by Kühne (1). It was confirmed by Langley (2), who observed also "that the destruction by sodium carbonate solutions goes on very much more slowly at 15°C. than at body temperature." Langley's experiments were carried out with neutralized filtrates of extracts of rabbit gastric mucosa made with 0.1 per cent HCl. He found no especial differences in the pepsins from the cat, dog, mole, newt, and snake. He mentions a possibility of frog pepsin being destroyed more rapidly than that of mammals. Biernacki (3) observed that the proteolytic activity of gastric juice was retained at a higher temperature in acid solution than at neutrality. This author found also that the resistance to destruction by heat was increased by the presence of peptones, and that a pure preparation was accordingly less stable than the pepsin in gastric juice. Grober (4) concluded from the average of a number of determinations that urinary pepsin heated with 0.25 per cent HCl was destroyed at 66°C., while pepsin from the same source, dissolved in water, was destroyed at 64°C. This difference he interpreted as indicating a chemical combination between the acid and the pepsin. The combination was thought to retard the hydrolysis of the pepsin. This mechanism was considered by Grober to be the cause of the destruction of pepsin by heat.

Michaelis and Rothstein (5) investigated the kinetics of the destruction of rennin and pepsin. They were unable to distinguish between these two properties of gastric juice preparations by their behaviour towards alkali. They derived an empirical differential equation in which the velocity of autodestruction was proportional to the fourth power of the hydroxyl ion concentration. They found that

at 25°C. and at 35°C., at reactions more acid than pH 6.0, the enzyme suffers no loss in potency, and that on approaching neutrality from the acid side the velocity of destruction suddenly becomes increasingly more rapid, until, in faintly alkaline solutions, it is too great to be measured. Michaelis and Rothstein found that the autodestruction at any given hydrogen ion concentration was not monomolecular. The velocity at constant hydrogen ion concentration in their experiments was proportional to the one and one-half power of the enzyme concentration. The destroyed pepsin was without influence upon the destruction of the remaining active enzyme, from which Michaelis and Rothstein concluded that the reaction is irreversible.

Northrop (6) found that pepsin solutions were most stable at 38°C. at a hydrogen ion concentration of 10^{-5}. "Neither the purity of the enzyme nor the anion of the acid used exerted any marked influence upon the rate of destruction, or on the zone of hydrogen ion concentration in which the enzyme was most stable."

The ionic nature of pepsin was suggested by Loeb in 1909 (7).

These studies of the autodestruction of pepsin are unanimous in their agreement upon the rapid destruction of the enzyme in acidities less than pH 6.0 The purpose of the investigation described below was to discover, if possible, the mechanism of the action of the OH ion. The method differed from that of other workers in that the amount of active enzyme present, at any given hydrogen ion concentration, was determined for zero time. This procedure is facilitated by the fact that the logarithms of the amounts of active enzyme present give a straight line when plotted against the time that the pepsin has been subjected to the hydroxyl ion concentration under consideration.

The zero values at 15°C. so obtained, plotted against pH, fall about a curve which represents the dissociation of a univalent compound with a value for pK of 6.85. The conclusion seems permissible that pepsin is such a univalent compound. This is in accordance with the conclusion of Northrop (8), attained by a totally different method, that pepsin is a univalent compound in the range of hydrogen ion concentrations from pH 1 to pH 7.

When the initial amount of enzyme is taken to be the extrapolated value found for active enzyme at zero time, results at 20°C. and 37°C.,

with those obtained at 15°C., showed that the autodestruction of pepsin is a monomolecular reaction. Michaelis and Rothstein assumed that at all hydrogen ion concentrations 100 per cent of the enzyme was active at zero time. This assumption, however, in the light of the experimental data given below seems to be unsound.

The products of the autodestruction of pepsin exert no inhibiting influence on the rate of subsequent autodestruction. This conclusion is drawn from the absence of any constant diminishing tendency in the values for K, and is similar to that of Michaelis and Rothstein.

The velocity of autodestruction is directly proportional to the hydroxyl ion concentration, when this is greater than pOH 7.7. This linear relationship is shown in Table V and Fig. 3.

It is difficult to interpret the variations in K over the whole range of hydroxyl ion concentrations at which the autodestruction was followed. The curve obtained may represent two different processes, or one process somewhat similar to the neutralization of a strong acid with a strong base.

The relation of autodestruction to ionization and the monomolecular decomposition suggest the following outline of the mechanism involved in the autodestruction of pepsin.

There are two independent processes, both resulting in irreversible loss of enzyme activity. The first is the ionization of the pepsin, with the two obvious possibilities of pepsin as either a weak acid or a weak base. If it is a weak acid, Fig. 2 represents the undissociated residue of the enzyme; and it follows that it is the undissociated form of the enzyme which is active in hydrolysis. If pepsin is a weak base, on the other hand, then the curve represents the degree of dissociation, and it is the pepsin ion which is active. For the purposes of discussion the enzyme will be considered as a weak acid and the curve will be taken to represent its undissociated and hydrolytically active residue. It is the ionized form, therefore, which must be considered to undergo the irreversible change which results in the destruction of the enzyme. This occurs at zero time, *i.e.* as soon as the alkali is mixed with the pepsin. The progressive falling off from 100 per cent, with increasing hydroxyl ion concentration, of the extrapolated values at zero time, makes it necessary to consider this change irreversible. Since ionization is completely reversible, another subsidiary reaction

must be considered to occur, possibly some stereoisomeric transformation, from which the original, undissociated hydrolytically active form, cannot be regained on restoration of the acidity to pH 1.6.

This transformation must permit the newly formed, irreversible compound to function in the ionic equilibrium as well as its precursor. Otherwise the second component of the process of autodestruction about to be discussed could not be defined by the equation for a monomolecular reaction.

The first process can be represented then by the following equations.

$$(1)\quad HA \rightleftarrows H^+ + A^-$$

$$(2)\quad A^- \rightarrow A_1$$

Equation (1) represents ionization and (2) the instantaneous, irreversible, complete conversion of the anion into an isomer, which, while it may participate in the ionic equilibrium, does not, on recombination with H^+, form active enzyme.

The second mode of irreversible loss of activity of the pepsin can be represented by the equation,

$$(3)\quad HA \rightarrow B$$

As discussed above, this reaction is defined by the mass law equation for a monomolecular reaction.

The original amount of active enzyme; *i.e.*, a, in the equation $K = \frac{1}{t} \log \frac{a}{a - x}$, is the extrapolated value of active enzyme at zero time.

This quantity is represented by the amount of H A at any given pH at zero time as shown in equation (1). If reaction (3) were dependent upon (1) and (2), then as H A was converted to B, equation (1) would be reversed in order to form H A from H^+ and A^-. If this happened then (3) could not be defined by the monomolecular equation.

The mechanism of this independence of (3) from (1) is probably represented by equation (2) which does not permit the reversion of any of A to H A. This possibility suggested; *viz.*, the formation of an isomer, which, while incapable of taking part in the reconstitution of the hydrolytically active form of the enzyme, may nevertheless func-

tion in ionic equilibrium, does not appear capable of direct experimental verification. A similar conception was advanced by Northrop (9).

The scheme involves two consequences. The first is, that no matter how low the temperature at which a solution of pepsin be maintained, if the C_{H^+} be lowered below that required for minimum ionization, there is an immediate and irreversible destruction of more or less of the pepsin depending on the pH. The second consequence is that the relation of the progressive autodestruction to the C_{OH^-} must be independent of the degree of dissociation of the pepsin, *i.e.* must show no relation to the dissociation curve. Both of the consequences are found in practice.

The first is demonstrated in the fact that an extrapolated value of 100 per cent is not obtained at any pH at which dissociation occurs. The second is indicated by the lack of any obvious relationship between Figs. 2 and 3.

EXPERIMENTAL.

In the experiments at 15°C., egg albumin (Merck) and scale pepsin (Wyeth) were employed. In those at 20°C., and at 37°C., which were performed 2 years previously, egg albumin (Merck) and scale pepsin (Merck) were used.

A 10 per cent solution of pepsin was made in N/10 HCl, giving an acidity approximating pH 3. An electrometric titration was carried out with N/1 NaOH to ascertain the amount of alkali required to bring the pepsin solution to a desired pH. For the autodestruction experiments 10 cc. of the stock 10 per cent pepsin solution was measured into a 50 cc. volumetric flask in a water bath at 15°C. Distilled water employed for dilution, and the N/10 NaOH, were also brought to 15°C. The calculated amount of alkali was added to the volumetric flask followed by distilled water up to the mark. The flask was shaken, and the contents transferred to a 100 cc. Erlenmeyer flask which was stoppered and placed in the water bath at 15°C. At the recorded times 5 cc. of this solution was removed with a pipette and added to 50 cc. of albumin solution at pH 1.6 and at 30°C. The hydrolysis was allowed to proceed for 1 hour when 50 cc. of the digest was removed and pipetted into 12.5 cc. of 10 per cent trichloroacetic

acid. The total nitrogen of the filtrate, after deducting the appropriate blanks, was taken as representing the amount of protein hydrolyzed. After allowing for dilution, the percentage of active enzyme was calculated from the amount of hydrolysis effected by 5 cc. of similar pepsin solution which had been diluted with water only, and to which no alkali had been added. Autodestruction was assumed to have commenced from the moment at which the NaOH was added to the pepsin.

The hydrogen ion concentration was measured both electrometrically and colorimetrically. M/10 phosphate solutions with methyl red, brom-cresol purple, and brom-thymol blue as indicators, were employed as colour standards. The colour of the pepsin solution was not deep enough to interfere with colorimetric estimations. Throughout the range from pH 5.5 to 7.15 the electrometric and colorimetric readings were identical.

On account of the relatively high concentration of albumin employed, 3.2 per cent, it was not necessary to add a buffer to the protein solutions undergoing hydrolysis. With the most active pepsin solution the pH, after 1 hour's hydrolysis at 30°C., rose only from 1.6 to 1.7.

Three controls were used for each hydrogen ion concentration; the estimation of total non-protein nitrogen of albumin and of pepsin, and the determination of the amount of hydrolysis effected by enzyme which had been diluted only with water, *i.e.* had undergone no autodestruction. In the results given in Table I and Fig. 1 these controls have been deducted. The amounts of active enzyme were calculated from the proportion between the amounts of hydrolysis obtained with the enzyme partially destroyed and that effected by enzyme of unimpaired activity.

At each C_{H^+} the extrapolated zero value ((a) in Table I) is therefore stated as a proportion of the original unimpaired activity of the enzyme. Similarly, the values given under $(a - x)$ (Table I) represent the amounts of hydrolysis obtained with partially destroyed enzyme. K (Table I) is calculated by means of the mass law equation for a monomolecular reaction. In Columns (a) and $(a - x)$, 1 per cent represents about 0.15 to 0.3 mg. of N in the macro-Kjeldahl determination.

In Fig. 1 are plotted the logarithms of the amounts of active enzyme

TABLE I.

The Effect of C_{H^+} on Autodestruction of Pepsin at 15°C.

pH	Time	Amount of active enzyme $(a - x)$	Amount of active enzyme at zero time (a)*	$K = \frac{1}{t} \log \frac{a}{a-x}$	Average value for K
	min.	*per cent of original amount*	*per cent of original amount*		
4.35	10	99	99.6	.0002	.0004
	20	96.3		.0007	
	40	96.0		.0004	
	50	94.7		.0004	
4.6	10	(96)	99	—	—
	20	100		—	
	30	98		—	
	40	98		—	
	50	100		—	
5.2	10	95	96	—	—
	20	(91)		—	
	30	96		—	
	50	(99)		—	
	60	96		—	
5.9	10	88.5	88.8	.0002	.0002
	20	(90)		—	
	30	86		(.0007)†	
	40	87.5		.0002	
	50	87.6		.0001	
5.9	10	82	80.9	—	.0005
	20	79		.0005	
	30	78		.0006	
	40	77.6		.0005	
6.22	10	80	80.7	(.0004)	.001
	20	77.5		.001	
	30	75.4		.001	
	40	73.6		.001	
	50	72.0		.001	
6.4	10	71	73.3	.0014	.0014
	20	69		.0013	
	30	66		.0015	

TABLE I—*Concluded.*

pH	Time	Amount of active enzyme $(a - x)$	Amount of active enzyme at zero time (a)*	$K = \frac{1}{t} \log \frac{a}{a - x}$	Average value for K
	min.	*per cent of original amount*	*per cent of original amount*		
6.54	10	68.3	67.9	—	.0007
	20	66.0		.0007	
	40	63.1		.0008	
	50	62.8		.0007	
6.64	15	57.7	58.2	(.0003)	.0014
	30	52.2		.0015	
	45	50.6		.0014	
	60	48.8		.0013	
6.75	10	46	56.2	.009	.011
	20	33.5		.011	
	30	23		.013	
	40	19		.012	
	50	16		.011	
6.85	10	52.2	69.3	.012	.012
	20	37.4		.013	
	30	28.7		.013	
	40	21.3		.013	
	50	18.8		.011	
6.97	4	29.6	44.2	.044	.045
	8	17.9		.049	
	12	12.9		.045	
	16	9.3		.042	
	20	5.3		.046	
7.15	2	18.3	26.3	(.08)	.17
	4	5.5		.17	
	6	2.5		.17	
	8	0.9		.18	
	10	0.6		.16	
7.35	1.25	4	23.2	.61	.66
	2	1		.68	
	3	0.2		.69	

* Obtained by extrapolation (Fig. 1).

† Bracketted values for K were not used in computing the average value for K.

present against the length of time that the pepsin solutions were subjected to the respective hydroxyl ion concentrations. These plots give straight lines, as mentioned above, and are produced to zero time.

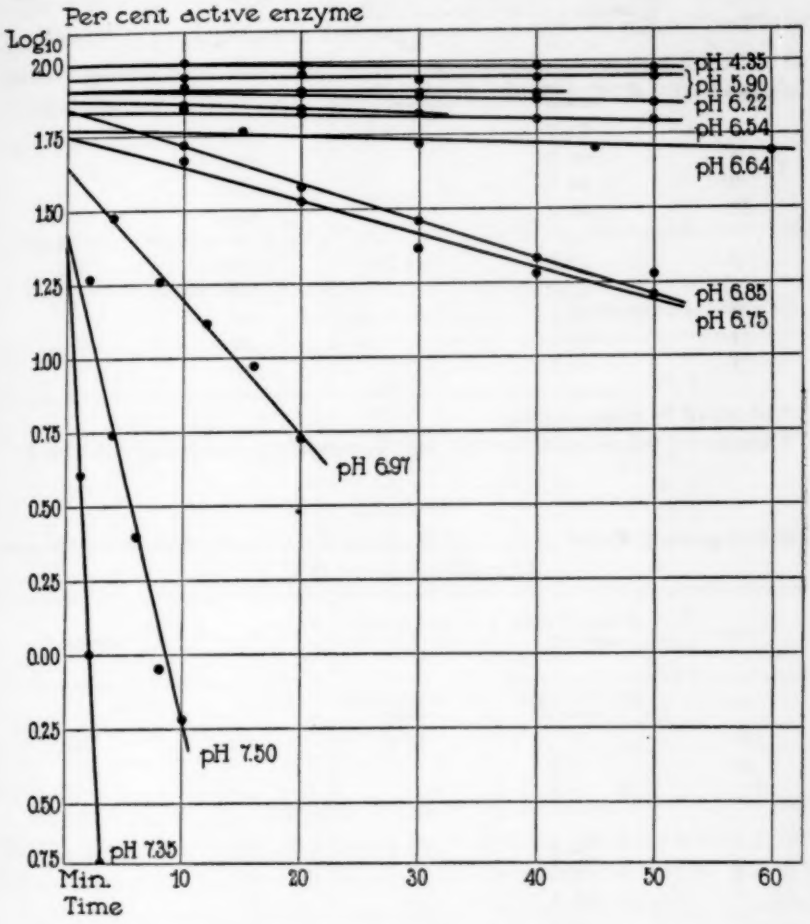

FIG. 1. The autodestruction of pepsin at 15°C. with time, at various reactions. The points are the $\log_{10}$ of amounts of active enzyme (in per cent of original amount). The values for the per cent of active enzyme at zero time are plotted against pH in Fig. 2.

In Tables II and III are given results of autodestruction experiments carried out at 20°C. and at 37°C. The pH values are uncertain and hence these cannot be compared with experiments at 15°C. They

TABLE II.

Autodestruction of Pepsin at Different Hydrogen Ion Concentrations More Alkaline than pH 6.0 and at 20°C.

Time	Amount of active enzyme $(a - x)$	Amount of active enzyme at zero time (a)*	$K = \frac{1}{t} \log \frac{a}{a - x}$	Average K
min.	*per cent of original amount*	*per cent of original amount*		
3	75.9	81.3	.010	.011
6	69.2		.011	
9	64.6		.011	
12	60.3		.011	
3	36.3	44.7	(.030)†	.041
6	25.1		.042	
9	19.1		.041	
12	15.1		.039	
15	10.5		.042	

* Obtained by extrapolation.

†Bracketted values for K were not used in computing the average value for K.

TABLE III.

Autodestruction of Pepsin at Different Hydrogen Ion Concentrations More Alkaline Than pH 6.0 and at 37°C.

Time	Amount of active enzyme $(a - x)$	Amount of active enzyme at zero time (x)*	$K = \frac{1}{t} \log \frac{a}{a - x}$	Average K
min.	*per cent of original amount*	*per cent of original amount*		
15	33	62.4	.018	.017
30	19.6		.017	
105	1.0		.017	
3	36.8	50.1	.045	.045
9	19.2		.046	
12	15.2		.043	
15	10.4		.046	
3	29.6	39.8	.043	.056
6	18.4		.056	
9	12.0		.058	
12	8.8		.055	
15	4.0		.067	

* Obtained by extrapolation.

show, however, that at a given pH the course of the autodestruction of pepsin at these temperatures is defined by the monomolecular equation as at 15°C.

In Table IV are given, for various hydrogen ion concentrations, the values for the undissociated residue of an acid with pK 6.85. These are compared with the extrapolated values of active enzyme at zero time at the same reaction. The data in Table IV are depicted graphically in Fig. 2.

TABLE IV.

Amounts of Active Enzyme at Zero Time Compared with the Dissociation of an Acid, pK 6.85.

pH	Active enzyme at zero time	Undissociated residue of acid
	per cent	*per cent*
4.35	99.6	99.7
4.6	99.0	99.0
5.2	96.0	97.8
5.9	80.9	89.9
5.9	88.8	89.9
6.22	80.7	81.0
6.4	73.3	73.8
6.54	67.9	67.1
6.64	58.2	61.9
6.75	56.2	55.7
6.85	69.3	50.0
6.97	44.2	43.2
7.15	26.3	33.4
7.35	23.2	24.0

In spite of the experimental difficulty of obtaining close agreement in the amounts of active enzyme at zero time, as evidenced by the disagreement in the results for two different experiments at pH 5.9 (Table I) and other discrepancies, the majority of the values obtained fall closely about the ideal dissociation curve (Fig. 2).

These discrepancies do not, in any case, affect the constancy of the value for K at any pH, though they may alter its absolute value, and are insignificant when the whole range of variation of K with pOH is considered.

In Table V are given the average values of K in Table I, the values

for pK, and the corresponding hydroxyl ion concentrations. By extrapolation from the data given by Michaelis (10) the value for pK_w at 15°C. was taken as 14.24. This value minus the pH gives the pOH.

In Fig. 3 are plotted the values of pK against pOH. The curve shows a direct proportionality between the velocity of autodestruction and C_{OH^-} in the range of concentrations greater than pOH 7.7; and possibly another proportionality, also direct, in the range from beyond pOH 9.89 to 7.7.

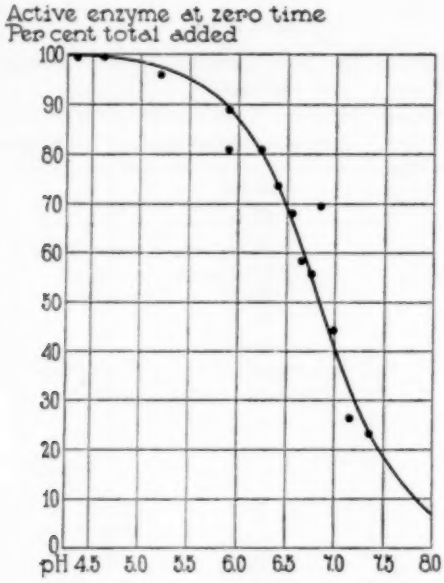

FIG. 2. The ideal dissociation curve of an acid with pK 6.85. The points are obtained from experimental data. They are amounts of active enzyme at zero time for the noted pH values and are obtained by extrapolation (Fig. 1).

Not all the experiments recorded in Table I were carried out with the same albumin solution. In those at pH 4.35, 5.9, 6.22, and 7.35 undenatured albumin was employed, while acid metaprotein served as substrate for the action of the pepsin in the experiments at pH 4.6, 5.2, one at 5.9, 6.4, 6.54, 6.64, 6.75, 6.85, 6.97, and 7.15. In the earlier experiments at pH 6.54, 6.64, 6.85, 6.97, and 7.15 the existence of a strict linear proportionality between the amount of hydrolysis in 1

hour at 30°C. and the concentration of pepsin was assumed. This assumption was made on the basis of results obtained by McFarlane, Dunbar, Borsook, and Wasteneys (11) with acid metaprotein as substrate. They found denatured albumin to give linear proportionality between velocity of hydrolysis and concentration of enzyme. It was found later that this result could not invariably be obtained. This was the case with the denatured albumin employed in the present experiments. An error is therefore introduced in the computations of results in the five experiments mentioned. It is believed, however,

TABLE V.

Relation of the Velocity of Autodestruction, K, to the Hydroxyl Ion Concentration.

pH	pOH	*K*	pK
4.35	9.89	.0004	3.40
4.6	9.64	—	—
5.2	9.04	—	—
5.9	8.34	.0005	3.30
5.9	8.34	.0002	3.70
6.22	8.02	.0010	3.0
6.4	7.84	.0014	2.85
6.54	7.70	.0007	3.15
6.64	7.60	.0014	2.85
6.75	7.50	.011	1.96
6.85	7.39	.012	1.92
6.97	7.27	.045	1.35
7.15	7.09	.17	0.77
7.35	6.89	.66	0.18

that it does not invalidate the conclusions, because under the experimental conditions the error introduced is not great.

Empirical reference curves were employed in calculating the amount of active enzyme in the majority of the experiments. The data for the reference curve for experiments at pH 4.35, 5.9, 6.22, and 7.35 are given in Table VI and the curve is shown in Fig. 4.

The procedure was as follows: varying amounts of 2 per cent pepsin were added to 50 cc. of 3.2 per cent undenatured albumin at pH 1.6. In order to maintain the nitrogen content the same with each concentration of enzyme the pepsin was diluted with an inactivated solution of the same concentration. The stock pepsin solution was

brought to pH 7.5 at room temperature for 10 minutes, then acidified to pH 1.6 and diluted to a final concentration of 2 per cent. The amount of hydrolysis at 30°C. for 1 hour was measured in the usual manner by precipitation of the unhydrolyzed protein with

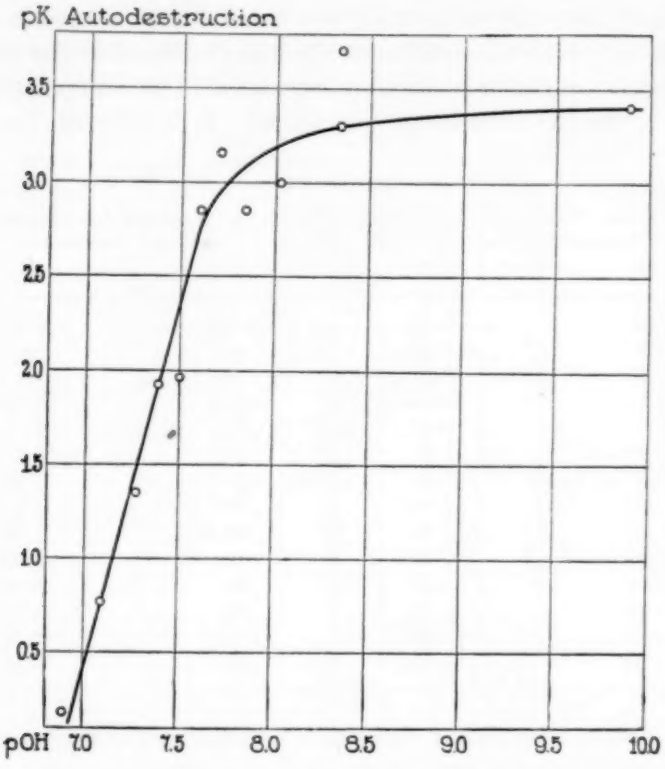

FIG. 3. The relation of the velocity of autodestruction to C_{OH^-} plotted as pK against pOH.

trichloroacetic acid, and estimation of the nitrogen content of the filtrate.

For experiments at pH 4.6, 5.2, one at 5.9, 6.4, and 6.75, acid metaprotein was used, and another reference curve.

The results obtained with the three solutions of albumin are incorporated into one body of data. The values for active enzyme at

zero time, whether obtained with acid metaprotein or with undenatured albumin fall near one dissociation curve as shown in Fig. 2. When

TABLE VI.

Relation between Amount of Hydrolysis in 1 Hour at 30°C. and the Concentration of Pepsin.

2 per cent active pepsin	2 per cent inactive pepsin	3.2 per cent albumin	Albumin N hydrolyzed
cc.	*cc.*	*cc.*	*mg.*
5	0	50	77.6
4	1	50	69.8
3	2	50	59.2
2	3	50	46.8
1	4	50	32.6

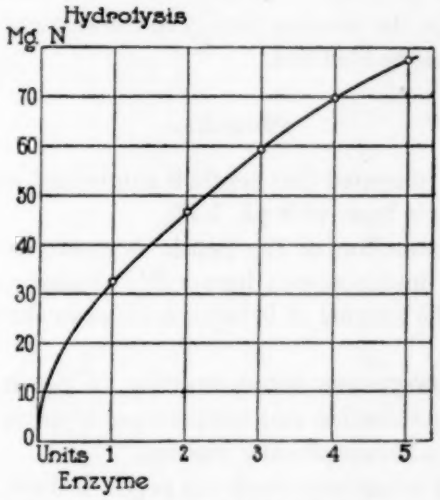

FIG. 4. A reference curve. The relation between the amount of hydrolysis (mg. of N) in 1 hour at 30°C. and the concentration of pepsin (cc. of 10 per cent solution).

the various quantities of active enzyme, calculated in each case according to the nature of the substrate, are inserted into the monomolecular equation, a practically constant value for K is obtained at

each hydrogen ion concentration. These values for K as Fig. 3 shows, vary, in general, with the hydroxyl ion concentration, regardless of the kind of albumin used in the particular experiment. These coincidences give added support for the validity of the methods employed for calculating active enzyme.

Observations made in the course of these experiments demonstrate the need for caution in comparing results obtained with different solutions of presumably the same albumin. The undenatured albumin in the experiments described here was hydrolyzed by a given solution of pepsin nearly three times as quickly as a similar solution of albumin which had been standing at pH 1.6 at room temperature for several weeks. Any relationship found between velocity of hydrolysis and enzyme concentration with a given solution of albumin may not be assumed to obtain with any other similar solution subjected to even slight changes in previous treatment. In order to obtain directly comparable results, the previous history of the albumin used for every determination must be identical.

SUMMARY.

1. Evidence is presented that pepsin is a univalent acid with a value for pK of 6.85 (or a base, with pK 7.39).

2. The autodestruction of the pepsin is shown to be dependent in part upon an instantaneous irreversible change occurring in the ionized form of the enzyme (if it be an acid) or in the unionized form (if it be a base).

3. A further progressive autodestruction of pepsin at any given hydrogen ion concentration and temperature is defined by the mass law equation for a monomolecular reaction

4. The velocity of autodestruction of pepsin is directly proportional to the hydroxyl ion concentration. It is much less in the range of hydroxyl ion concentration from pOH 9.89–7.7, than in the range greater than pOH 7.7. In both of these ranges variations in pK with pOH may be represented by straight lines.

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BIBLIOGRAPHY.

1. Kühne, W., *Verhandl. naturh.-med. Ver. zu Heidelberg*, 1877, l, 193.
2. Langley, J. N., *J. Physiol.*, 1880–82, iii, 246.
3. Biernacki, E., *Z. Biol.*, 1891, xxviii, 49.
4. Grober, J. A., *Arch. exp. Path. u. Pharmakol.*, 1904, li, 103.
5. Michaelis, L., and Rothstein, M., *Biochem. Z.*, 1920, cv, 60.
6. Northrop, J. H., *J. Gen. Physiol.*, 1919–20, ii, 465.
7. Loeb, J., *Biochem. Z.*, 1909, xix, 534.
8. Northrop, J. H., *J. Gen. Physiol.*, 1924–25, vii, 603.
9. Northrop, J. H., *J. Gen. Physiol.*, 1919–20, ii, 471.
10. Michaelis, L., Die Wasserstoffionenkonzentration, Berlin, 2nd edition, 1922, 23.
11. McFarlane, J., Dunbar, V. E., Borsook, H., and Wasteneys, H., *J. Gen. Physiol.*, 1926–27, x, 437.

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